Universidade Nova de Lisboa

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Different Models of DNA Immunization as Strategy for Vaccine Development Against African Trypanosomiasis

Adriana Beatriz Oliveira Temporão

DISSERTATION TO OBTAIN THE MASTER'S DEGREE IN BIOMEDICAL SCIENCES WITH SPECIALIZATION IN MOLECULAR BIOLOGY IN TROPICAL AND INTERNATIONAL MEDICINE

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Author: Adriana Beatriz Oliveira Temporão

Supervisor: Marcelo Sousa Silva, Assistant Researcher

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If you can dream it, you can do it.
Walt Disney

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Abbreviations List

APC - Antigen Presenting Cells

BBB - Blood-Brain Barrier

BGH - Bovine Growth Hormone

CMV - Cytomegalovirus

CNS - Central Nervous System

CRD - Cross-Reacting Determinant

CSF - Cerebrospinal Fluid

DC - Dendritic Cells

DNA - Desoxiribonucleic acid

EDTA - Ethylenediamine tetraacetic acid

ELISA - Enzyme-Linked Immunosorbent Assay

GPI - Glycosylphosphatidylinositol

HAT - Human African Trypanosomiasis

HBV - Hepatitis B virus

HIC - Hydrophobic Interaction Chromatography

HIV - Human Immunodeficiency Virus

HPV - Human Papillomavirus

HRP - horseradish peroxidase

IL - Interleukin

ISG - Invariant Surface Glycoprotein

mfVSG - membrane form VSG

MHC - Major Histocompatibility Complex

MSP - Major Surface Protease

NO - Nitric Oxide

OPD - o-phenylenediamine dihydrochloride

PAMP - pathogen- associated molecular pattern

PBS - phosphate buffered saline

PCR - Polymerase Chain Reaction

PLC - Phospholipase C

SDS - sodium dodecyl sulfate

SDS-PAGE - SDS-polyacrylamide gel electrophoresis

SIF - Stumpy Induction Factor

sVSG - soluble VSG

TAE - Tris-Acetate-EDTA

TLR - Toll-Like Receptors

TNF - Tumor Necrosis Factor

TSA - *Trans*-sialidase

TrV - Triatoma virus

VLP - Virus-Like Particles

VSG - Variant Surface Glycoprotein

Abstract

African Trypanosomiasis, also known as sleeping sickness, caused by the protozoan *Trypanosoma brucei*, is a neglected tropical disease. This disease can be successfully controlled, as has been proven in the past; nevertheless, the growing number of people affected and at risk makes the development of a vaccine a priority. *T. brucei* is capable of constantly evading the host immune system, due to a remarkable mechanism of defense, which provides a great antigenic variation. Due to this mechanism it has been very difficult to develop an effective vaccine. However, new approaches have been pursued, one of which, the vaccination strategy with plasmid DNA has revealed some promising results. Based on this, this work aims to use three immunization strategies: the first one, DNA vaccination, using two plasmids DNA, encoding antigenic candidates from *Trypanosoma brucei*; the second one, using these antigenic candidates together with a nanoformulation; and the third one, using VLPs (Virus-Like Particles). The three models used in the development of DNA vaccines against *T. brucei* use two important proteins of the parasite: MSP (Major Surface Protease) and PLC (Phospholipase C). MSP is a surface zinc metalloprotease that is believed to be responsible by the release of a VSG (Variable Surface Glycoprotein) fragment. PLC is a phospholipase anchored to a GPI (Glycosylphosphatidylinositol) residue that cleaves a full-length VSG protein from the cell surface. As we can see, both proteins are responsible by the VSG release, by the normal differentiation from bloodstream to procyclic form, and they participate synergistically in VSG loss during differentiation.

After immunization with the first two strategies, although the titres were low, mice produced antibodies anti-*Trypanosoma brucei brucei*. The animals that presented a better immune response were the ones immunized with the mix of plasmids together with the nanoformulation. Regarding the third model of immunization, the design of the VLPs was made, and the next step is evaluating them biologically.

Keywords: Sleeping Sickness, Human African Trypanosomiasis, *Trypanosoma brucei*, DNA Vaccines, VLPs.

Resumo

A tripanosomose Africana, também conhecida como Doença do Sono, causada pelo protozoário *Trypanosoma brucei*, é uma doença tropical negligenciada. Esta doença pode ser controlada, tal como foi provado no passado; no entanto, o crescente número de pessoas afectadas e em risco torna o desenvolvimento de uma vacina uma prioridade. *T. brucei* é capaz de evadir constantemente o sistema imunitário do hospedeiro, devido ao seu extraordinário mecanismo de defesa, que lhe proporciona uma grande variação antigénica. Devido a este mecanismo de defesa tem sido muito difícil de desenvolver uma vacina eficaz. Contudo, têm sido procuradas novas técnicas, entre elas, uma estratégia de vacinação com DNA plasmídico que têm revelado resultados promissores. Tendo em conta estes resultados, este trabalho tem como objectivo o uso de três estratégias de imunização: a primeira, recorrendo a vacinas de DNA, usando dois plasmídeos que codificam candidatos antigénicos de *Trypanosoma brucei*; a segunda, usando estes candidatos antigénicos conjugados com uma nano-formulação; e a terceira, usando VLPs (*Vírus-Like Particles*). Os três modelos usados no desenvolvimento de vacinas de DNA contra *T. brucei* recorreram ao uso de duas importantes proteínas do parasita: a MSP (*Major Surface Protease*) e a PLC (*Phospholipase C*). A *MSP* é uma metaloprotease de zinco de superfície, que se acredita ser responsável pela libertação de um fragmento de VSG (*Variable Surface Glycoprotein*). A PLC é uma fosfolipase, ancorada a um resíduo de GPI (*Glycosylphosphatidylinositol*), que cliva integralmente uma proteína de VSG da superfície da célula. Como se pode ver, ambas as proteínas são responsáveis pela libertação das VSG, pela normal diferenciação da forma procíclica para a forma de corrente, e participam de forma sinérgica para a perda de VSG durante a diferenciação.

Após a imunização com as duas primeiras estratégias, apesar de em baixos níveis, os murganhos produziram anticorpos anti-*Trypanosoma brucei brucei*. Os que apresentaram melhor resposta imunológica foram os imunizados com a mistura de plasmídeos conjugados com a nano-formulação. Em relação ao terceiro modelo de imunização, o desenho das VLPs foi efectuado, e o próximo passo é a avaliação biológica das mesmas.

Palavras-chave: Doença do sono, Tripanossomose Africana Humana, *Trypanosoma brucei*, Vacinas de DNA, VLPs

1. Introduction

1. Introduction

Human African Trypanosomiasis (HAT), also known as, sleeping sickness, is caused by the protozoan parasite *Trypanosoma brucei*, and it is transmitted by the bite of tsetse flies (*Glossina spp.*). There are two subspecies of *T. brucei* that are pathogenic for humans and cause very distinct pathologies: *T. b. gambiense* and *T. b. rhodesiense* (WHO 2015a). It is estimated that 69.3 million people, in 36 sub-Saharan Africa countries, are at risk of having sleeping sickness, 57 million of which are at risk of getting *T. b. gambiense* and 12.3 million *T. b. rhodesiense* (Simarro *et al.* 2012; WHO 2015a). The first descriptions of this disease date back to the colonial period and were made by ship doctors and medical officers (WHO 2015b).

T. b. gambiense is found in Western and Central Africa (Figure 1A), and causes a chronic form of HAT that can have a duration of about 3 years (Checchi *et al.* 2008). On the other hand, *T. b. rhodesiense*, can be found in Eastern and Southern Africa (Figure 1B), causes an acute and fulminant form of the disease, which can lead to death within weeks or months (Odiit *et al.* 1997).

There is a third subspecies of *Trypanosoma brucei* parasites: *Trypanosoma brucei brucei*, which causes African Animal Trypanosomiasis and does not infect humans, just wild and domestic animals, such as donkeys, horses, goats, camels, mules, sheep, antelope. This disease is chronic and sometimes fatal in cattle, however is rarely fatal in swine. In animals, the first symptoms are lymphadenopathy, intermittent fever, anemia and progressive emaciation. The animal disease can vary between acute or chronic depending on the species, the age of the host and the parasite load (Acha & Szyfres 2003). This subspecies is widely used in experimental models of animal and human African Trypanosomiasis (de Sousa *et al.* 2010; Lança *et al.* 2011).

HAT was close to elimination during the 1960s, due to the establishment of systematic screening, treatment, and follow-up of patients for the *T. b. gambiense* HAT, and control of reservoir animals and vectors, in Eastern and Southern Africa, for the *T. b. rhodesiense* HAT (Simarro *et al*. 2011). The rarity of cases resulted in a lack of interest in disease surveillance and a decrease in disease control, which led to the re-

emergence in the 1980s. This re-emergence reached epidemic proportions, most of the infections being caused by *T. b. gambiense* (Holmes 2014).

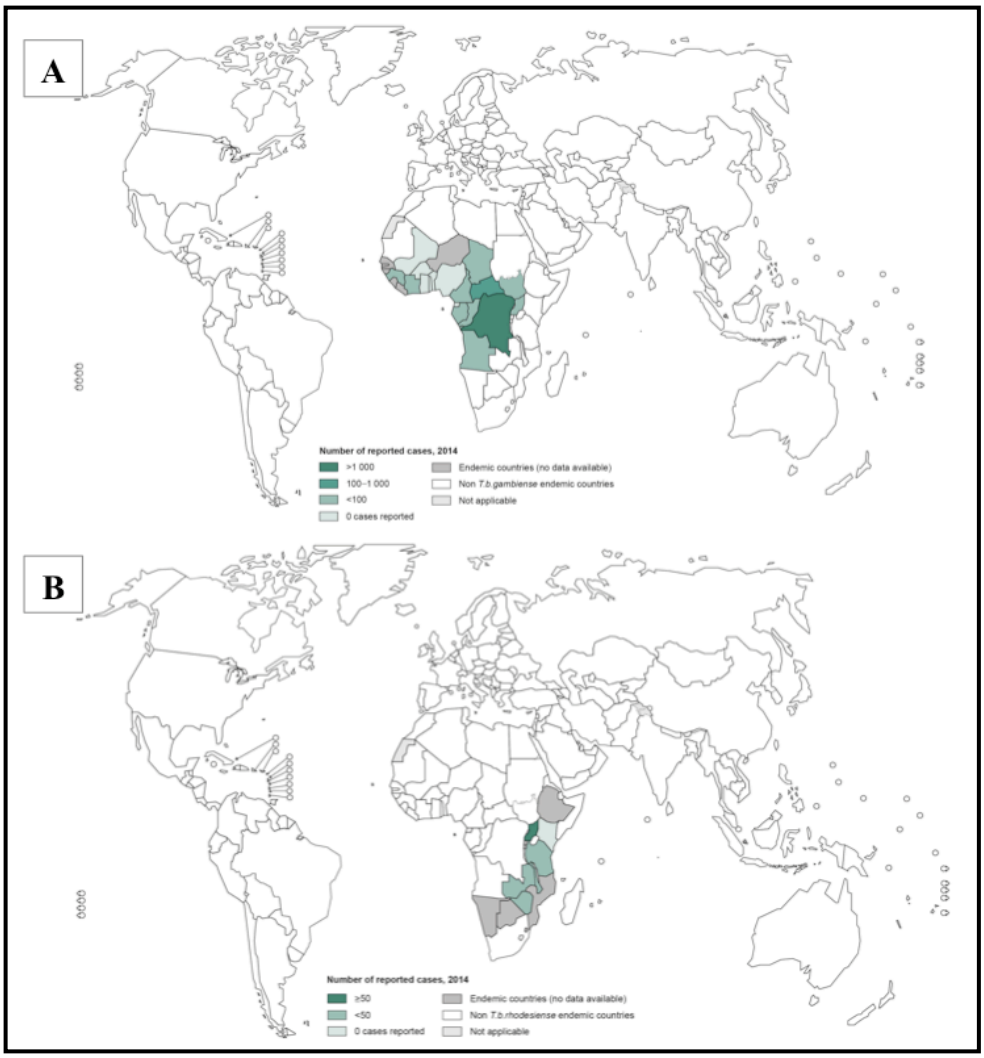

Figure 1: Distribution of Human African Trypanosomiasis: **A-** Distribution of Human African Trypanosomiasis (*T. b. gambiense*) (WHO 2015c); **B-** Distribution of Human African Trypanosomiasis (*T. b. rhodesiense*) (WHO 2015d).

Due to more health care facilities doing screening and the improvement of the diagnostic tests, in 2009, the number of new cases of HAT fell below 10.000 and the number of people screened increased (Simarro *et al.* 2011).

1.1 Pathology, diagnosis and treatment of Human African Trypanosomiasis

HAT has two different stages, being the clinical signs slightly different between *T. b. rhodesiense* and *T. b. gambiense*. However, they have one feature in common, if left untreated they can lead to coma and death.

After inoculation, the trypanosome multiplies at the site of infection, producing a local skin reaction, called chancre. This reaction is more common in *T. b. rhodesiense* than in *T. b. gambiense*, and is the first symptom of this disease (reviewed in Barrett *et al.* 2003).

The first stage of the disease starts when the parasites reach the draining lymph nodes and the bloodstream; this stage goes by the name of haemolymphatic stage. The commonest characteristic symptom of this stage is the irregular fever, which reflects the parasitemia levels in the blood. Other symptoms, like headache, pruritus and lymphadenopathy are also seen. However, there are some differences between East and West African trypanosomiasis. In the first one, the symptoms, even at the first stage of the disease, can be severe, if there is no quick access to treatment, patients can die. On the other hand, in the infection caused by *T. b. gambiense* the most common signs of the disease are lymphoadenopathy (Winterbottom's sign), hepatosplenomegaly and faint rash (reviewed in Stich *et al.* 2002; Barrett *et al.* 2003; Brun *et al.* 2010).

As the disease progress to the second stage, also known as, meningoencephalitic stage, the parasites cross the blood-brain barrier (BBB), invading the central nervous system, causing progressive neurological damage. In *T. b. rhodesiense*, this happens within a few weeks of infection, unlike *T. b. gambiense*, which takes between several months to years. The main symptom of the second stage is the sleep disorder; this is characterized by deregulation of the circadian rhythm of the sleep cycle and fragmentation of the sleeping pattern. Other neurological symptoms are tremor, fasciculation, general motor weakness, paralysis of a limb, hemiparesis, akinesia, and abnormal movements such as dyskinesia or chorea-athetosis. Difficulty in concentration, reduced higher mental functions, are other symptoms which culminate in a final state of sleepiness (reviewed in Stich *et al.* 2002; Barrett *et al.* 2003; Brun *et al.* 2010).

This disease needs an accurate and early diagnosis. Control programmes use a three-step approach: screening, diagnostic confirmation and staging. The diagnostic method may vary between the two forms of the disease.

The card agglutination test for trypanosomiasis - *T. b. gambiense*, used in the screening step, is a specific and, most of all, fast and practical serological test, that allows hundreds of people to be tested daily. Nevertheless, this method has limitations: high frequency of equivocal results and a limited sensitivity. Other serological tests used to screen, are immunofluorescence and enzyme-like immunosorbent assays (ELISA) (Noireau *et al.* 1988; Lejon *et al.* 1998). However, in addition to all necessary equipment, these techniques are time-consuming, making them mainly used in non-endemic countries, where the number of samples are lower and the equipment already exists. Even though the serologic test comes back negative, in patients who have had recent infection or a clinical suspicion of sleeping sickness, health professionals, are advised to search for parasites in the blood. For *T. b. rhodesiense*, there is no serological screening test. In this case, screening step has to be done, based on non-specific clinical presentation and history of exposure (reviewed in Brun *et al.* 2010).

For diagnostic confirmation, a microscopic examination of lymph node aspirate and blood, or both, is needed. It might be needed a concentration step, to increase the sensitivity of this method (Lutumba *et al.* 2007). PCR (Polymerase Chain Reaction) on blood has 99% sensitivity and 97.7% specificity, but, usually, is impracticable in endemic regions, because of the advanced facilities required to perform this method (Mugasa *et al.* 2012).

In the diagnosis process it is essential to differentiate the stage of the disease, by examination of the cerebrospinal fluid (CSF) after lumbar puncture, due to the pharmacological differences between the two stages. According to the WHO, the presence of trypanosomes in the CSF or a white blood cell count of more than 5 cells per μL, or both, defines the late-stage disease (Noireau *et al.* 1991). The measurement of IgM concentrations, in the CSF, is a useful adjunct to the second-stage diagnosis (Lejon *et al.* 2002).

There are some non-invasive staging methods, like, polysomnography and actigraphy; however, they are not used as a basis for guiding the initial treatment. Polysomnography identifies alterations of sleep structure that occur in the late-stage HAT (Buguet *et al.* 2005). Actigraphy uses an actigraph on the wrist to measure body movement, and can detect the sleep/wake cycle abnormalities (Njamnshi *et al.* 2012).

The treatment of sleeping sickness is based on the disease stage and on the subspecies of trypanosome. There are few drugs available to treat this condition.

First-stage *T. b. gambiense* HAT is treated with intramuscular pentamidine. This drug, although effective has many possible complications, as hyperglycaemia or hypoglycaemia, prolongation of the QT interval on electrocardiogram, hypotension and gastrointestinal features. The treatment for early-stage *T. b. rhodesiense* HAT is suramin, usually administered intravenously, which has as potential complications renal failure, skin lesions, anaphylactic shock, bone marrow toxicity and neurological complications (reviewed in Babokhov *et al.* 2013).

There is only one drug effective in treating second-stage Eastern HAT, melarsoprol. Nevertheless, this drug is very toxic, injections are exceedingly painful, and there are records of treatment failures due to resistance. Melarsoprol treatment can result in reactive encephalopathy, agranulocytosis, skin rashes, peripheral neuropathy and cardiac arrhythmias. Eflornithine is the drug used to treat late-stage *T. b. gambiense*. This drug can cause bone marrow toxicity, alopecia, seizures and gastrointestinal symptoms, and there is a potential for drug resistance in the affected areas in Africa (reviewed in Kennedy 2013).

Two new drugs for second-stage *gambiense* HAT, Fexinidazole and SCYX-7158, are being evaluated in clinical trials (Jacobs *et al.* 2011; Tarral *et al.* 2014). There is a study being conducted using melarsoprol, where it was changed the delivery method by constructing melarsoprol-cyclodextrin inclusion complexes, and it is a promising candidate to treat HAT (Rodgers *et al.* 2011).

1.2 *Trypanosoma brucei*

1.2.1 Life cycle of *Trypanosoma brucei*

Trypanosoma brucei is a heteroxenic protozoan parasite from the Trypanosomatidae family and the Kinetoplastida order (reviewed in Simpson *et al.* 2006). There are four main developmental stages in the entire life cycle of *T. brucei*: epimastigotes, procyclic forms, slender metacyclic trypomastigotes, and stumpy metacyclic trypomastigotes (Figure 2).

Once an infected tsetse fly takes a blood meal it injects stumpy metacyclic trypomastigotes into the mammalian host. The metacyclic trypomastigotes differ into proliferative, slender, trypomastigotes that can multiply themselves by binary fission throughout the body fluids, such as lymph and cerebrospinal fluid. During the whole life cycle in the mammalian host, *T. brucei* is in the extracellular space. When a tsetse fly bites an infected mammal, ingests bloodstream trypomastigotes that differentiate into proliferative procyclic trypomastigotes in the tsetse midgut and transform into epimastigotes. Then, in the salivary glands, they multiply and transform into non-proliferative, stumpy, metacyclic trypomastigotes. Only then, the trypanosomes are ready to infect another host, once the *Glossina* takes a blood meal (D'Avila-Levy *et al.* 2014).

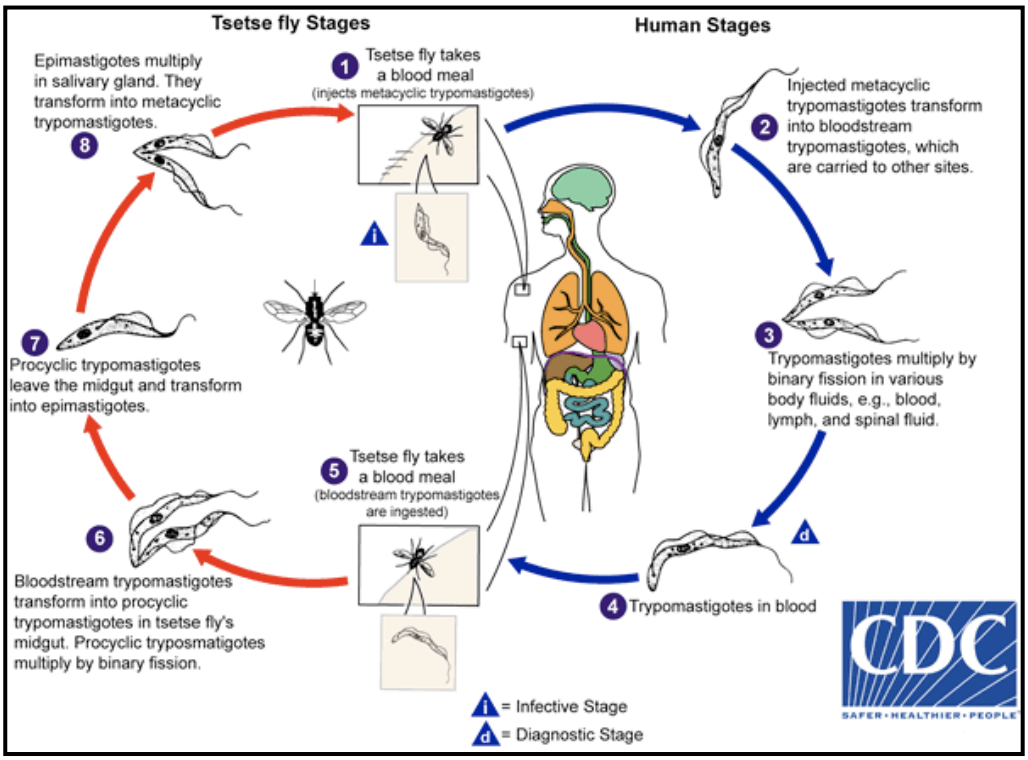

Figure 2: Schematic illustration of the life cycle of *Trypanosoma brucei* (CDC 2015).

As the parasitemia increases, in the vertebrate host, so does the density (Figure 3), which induces the parasites to produce a trypanosome factor, the stumpy induction factor (SIF). SIF stimulates the transition from slender forms to stumpy forms. These forms differ in terms of capacity to proliferate – slender forms proliferate, while stumpy forms are arrested; nevertheless, both express the bloodstream stage-specific antigen, variant surface glycoprotein (VSG). The fact that stumpy forms are cell-cycle arrested combined with an elaborated mitochondrial activity, makes them pre-adapted to life in the tsetse vector (Vassella *et al.* 1997; Tyler *et al.* 1997). The establishment of parasite numbers in the blood and the immune evasion, through antigen variation, is due to the proliferation of slender forms. On the contrary, stumpy forms control the expansion of parasite numbers in the mammalian host bloodstream and consequently prolonging the host survival, increasing the probability of disease transmission (Tyler *et al.* 2001; Mony *et al.* 2014).

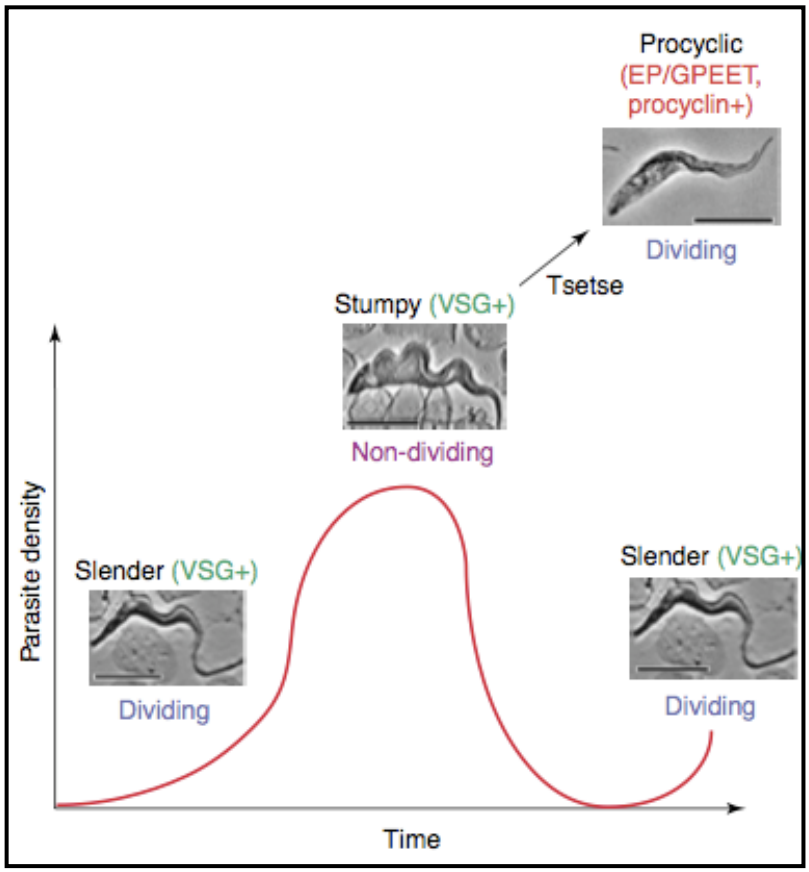

Figure 3: Life cycle stages in *Trypanosoma brucei* (Matthews *et al.* 2004).

African trypanosomes have at least three different survival strategies that are responsible for the persistent infection. The first one, is the antigenic variation of the surface coat (Cross 1996); second one, it is observed a repression of stimulatory components that results in immunosuppression by unknown mechanisms (reviewed in Donelson *et al.* 1998); and at least, clearance of surface-bound antibodies by the parasite motility. However, this clearance is, only effective when the antibody concentrations are low, it is not sufficient to protect the cells at high antibody titers (Engstler *et al.* 2007). It is believed, that the antigenic variation and the immunosuppression are the reason for the long-term persistence of an infecting population, and that the clearance of surface-bound immunoglobulin may contribute for the survival of individual cells during the emergence of a specific humoral immune response.

1.2.2 Cell structure of *Trypanosoma brucei* parasites

The elongated trypanosome cell (Figure 4) is composed of a highly polarized microtubule cytoskeleton, which gives the cell the characteristic shape and maintains it intact throughout the cell cycle (Sherwin & Gull 1989). The flagellar pocket is positioned at the posterior end of the cell. This structure is the exit point for the flagellum and is responsible by all the processes of endo and exocytosis of the parasite (reviewed by Overath & Engstler 2004). The motility of the *T. brucei* depends of its single flagellum, a semi-rigid structure found in the Kinetoplastids (Bastin *et al.* 1998; Vaughan & Gull 2003). Nowadays, the flagellum is recognized as the main contributor to the pathogenicity of these protozoa. In addition to the motility, it contributes to the recognition of the host/vector environment and promotes the recognition and attachment necessary for immobilizing the parasite at the vector surfaces at some life-cycle stages (Vaughan & Gull 2003). The flagellum begins in the basal body, which in turn, is connected through the mitochondrial membrane to the mitochondrial genome which is contained by the kinetoplast. A tripartite attachment complex links the basal body and the kinetoplast, and has to cross the cell and the mitochondrial membranes (Ogbadoyi *et al.* 2003).

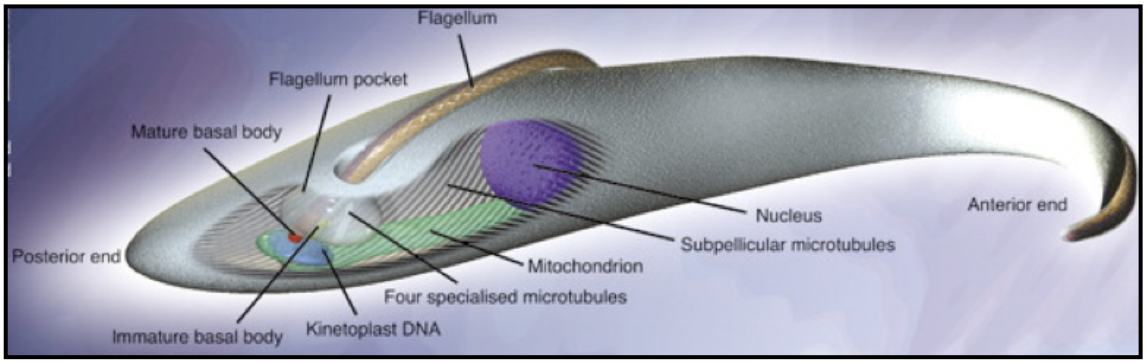

Figure 4: Schematic drawing of the *T. brucei* structure (Vaughan & Gull 2003).

1.2.3 The Variant Surface Glycoprotein Coat of *Trypanosoma brucei*

One of the main features of the *T. brucei* that raises interest between the scientific communities, is his capability of evading the host immune system, and thereby remains in circulation for months to years. This is achieved by antigenic variation of the parasite coat. The *Trypanosoma brucei* has a 15 nm Variant Surface Glycoprotein (VSG) coat that covers the entire cell, during the bloodstream form (Vickerman 1969). The VSG coat, as the name suggests, is composed by, approximately, 10 million copies of VSG molecules, and represents up to 10% of the total cell protein (Cross 1996).

The VSG monomer consists of an exposed N-terminal domain, comprising about ≈ 350 residues, that contains the biologically relevant epitopes (Hsia *et al.* 1996) and a membrane-proximal C-terminal domain, of 50 to 100 residues, that is inaccessible to the antibodies (Johnson & Cross 1979; Schwede *et al.* 2011). The C-terminal is attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor (Ferguson *et al.* 1988).

Over the course of the infection, the host immune system develops a response against the VSG expressed at that time, however this is inefficient in eradicating the entire parasite population, as some individuals have already switched their VSG (Vickerman 1978). The VSG coat also serves to protect less variable or even invariant surface proteins from immune effectors (Hutchinson *et al.* 2003).

There are two more life cycle steps where the VSG is shed from the parasite surface: when all cells exchange the metacyclic VSG for a bloodstream form VSG, within a few days after entry into a mammalian host (Esser & Schoenbechler 1985); and when the trypanosome enters the tsetse midgut. The procyclic form stops expressing any VSG and starts to express another GPI-anchored glycoprotein, yet invariant, the procyclin (Figure 3). When the parasites re-enter the vertebrate host as metacyclic trypomastigotes, the expression of VSG is activated once again (Bülow *et al.* 1989).

When the VSG coat is shed in the differentiation from stumpy bloodstream forms to procyclic forms, in the tsetse midgut, two forms of VSG were identified, *in*

vitro, within hours of the initiation of differentiation: one is full-length VSG protein that is cleaved from the cell surface by a GPI-phospholipase C (GPI-PLC) *(*Ziegelbauer *et al.* 1993) and the other, is a truncated VSG fragment released by proteolysis, by the major surface protease (MSP) (Bangs *et al.* 1997; Gruszynski *et al.* 2006).

1.2.4 Major Surface Protease and Phospholipase C from *Trypanosoma brucei*

There is a parasitic protozoa evolutionary related with *T. brucei*, that occurs as an extracellular form in the sand fly vector and as an intracellular form in their mammalian hosts macrophage, the *Leishmania spp*. These parasites contain a major surface zinc metalloprotease (MSP, also called GP63), which has been extensively studied. Among other function, this metalloprotease provides resistance to complement-mediated lysis before *Leishmania* enters the macrophage, participates in the attachment and entry into the macrophage supporting survival in the macrophage after entry (Brittingham *et al.* 1995; reviewed in Yao *et al.* 2003).

Previously, LaCount *et al.* reported the presence in the genome of the African trypanosome, of at least, three gene families (TbMSP-A, -B, and -C) encoding homologues of the GP63 found in *Leishmania* spp. The TbMSPs share about 33% sequence identity, positional conservation of 20 cysteines and 10 prolines, and a metalloprotease catalytic site motif, HEXXH, with *Leishmania* GP63, which suggests common three-dimensional features. All of these three families are expressed in bloodstream stage trypanosomes, but only TbMSP-B is found in procyclic stage. Although, they all are expressed in the bloodstream stage, the TbMSP-A mRNA is about twice as abundant as the TbMSP-B mRNA, which is four times more than TbMSP-C mRNA. The sequences from the three TbMSP share about 33% identity, and the major difference is in their termini. The TbMSP-A has an extended C-terminal region, rich in serines and glutamates, that was not seen in the other two, and finishes in a short hydrophobic segment. TbMSP-B has a hydrophobic tail in the C-terminal. TbMSP-C has a C-terminal region highly hydrophilic, rich in charged amino acids and

prolines, which indicates that it is not linked to a membrane via GPI anchor, unlike TbMSP-A and -B (LaCount *et al.* 2003).

It has been shown that TbMSP is a surface-localized zinc metalloprotease, with 60 kDa, that is expressed, mainly, in differentiating bloodstream to procyclic parasites and in established procyclic cells. This MSP, in concert with PLC, participates in the removal of the VSG coat when bloodstream stage trypanosomes differentiate to procyclic trypanosomes. A TbMSP-B$^{-/-}$PLC$^{-/-}$ double mutant cell line, failed to differentiate into the procyclic form, most VSG remaining in the cell surface. This cell line also exhibited an altered morphology: enlarged with multiple nuclei and kinetoplast, and detached flagellae. However, the single mutant cell line, TbMSP$^{-/-}$ and PLC$^{-/-}$, showed a delay in VSG loss, but they were still able to differentiate, which leads to the conclusion, that MSP and PLC act synergistically in the VSG loss during differentiation (Grandgenett *et al.* 2007).

A further detailed analysis provides evidence that TbMSP-B and GPI-PLC expression is coordinately and inversely regulated during differentiation (Gruszynski *et al.* 2006).

Another possible function of the MSP, it is related to the capacity of the *Trypanosoma brucei* in disrupting the blood-brain barrier and invade the central nervous system (CNS). It has been shown that the metalloproteases expressed on *T. b. brucei* bloodstream forms display hydrolytic activity on proteins like, gelatin, casein, and matrix proteins such as collagen (de Sousa *et al.* 2010).

Trypanosoma brucei has also an endogenous PLC of 39-40 kDa anchored to GPI residue, this protein does not have a N-terminal signal peptide nor a trans-membrane domain, it behaves as an integral membrane protein (Bülow & Overath 1986; Fox *et al.* 1986; Carrington *et al.* 1989). It is capable of hydrolysing the GPI anchor of the VSG, releasing dimyristyl glycerol (Ferguson *et al.* 1985). This hydrolysis results in the conversion of the hydrophobic membrane form of VSG (mfVSG) to a water soluble VSG (sVSG), resulting then in the shedding of the VSG from the parasite membrane. This reaction can be detected immunologically by detecting an epitope

contained in the residue of the anchor attached to the VSG, the cross-reacting determinant (CRD) (de Almeida & Turner 1983; Zamze *et al.* 1988).

GPI-PLC concentrates in the flagellar membrane, disposed in a patchy, linear array along the flagellar attachment zone and extending into the free flagellum and it remains in the same location, both before and after activation, which means that to cleave the GPI anchor of VSG it does not move from its location (Hanrahan *et al.* 2009; Sunter *et al.* 2013).

The GPI-PLC is only present in metacyclic and bloodstream stages of the life cycle, procyclic trypanosomes are resistant to this protein, because it contains GPI anchors bearing an extra acylation on the inositol ring (Field *et al.* 1991).

Although GPI-PLC does not act alone in the VSG release, as explained before, it is described as a virulence factor. In a cell line with the PLC gene deleted, the infection was able to proliferate, however the levels of parasitemia were lower when compared to a control cell line, and the survival times of the mice were longer (Webb 1997).

GPI-PLC is unable to release VSG of another parasite, when expressed by one trypanosome only targets its own plasma membrane. Also, VSG is not the only protein cleaved by GPI-PLC, this protein also acts in other proteins of lower and higher molecular mass, like other GPI biosynthetic intermediates (Cardoso De Almeida *et al.* 1999).

The action of GPI-PLC can be inhibited by some sulphydryl reagents, like Zn^{2+} and *p*-chloromercurylphenylsulphonic (Carnall *et al.* 1997).

1.3 Immunobiology of African Trypanosomiasis

Being an extracellular parasite, *T. brucei* encounters both innate and adaptive immune response from the host. As soon as the parasite enters in the host bloodstream it encounters the innate immune system as first barrier. The first response of the host to control the first peak of parasitemia consists of classical activated macrophages which phagocyte antibody-opsonised parasites (Shi *et al.* 2004) and secret pro-inflammatory

and trypanotoxic molecules, such as Tumoral Necrosis Factor - alpha (TNF)-α and nitric oxide (NO) (Mabbott *et al.* 1994; Sternberg & Mabbott 1996; Kaushik *et al.* 2000; Pan *et al.* 2006). In addition, these macrophages can be activated by the trypanosome DNA (Desoxiribonucleic acid) (Harris *et al.* 2006) or by the GPI anchor of the VSG (Coller *et al.* 2003).

Although initial inflammatory response is beneficial to the host at an early stage, a sustained inflammation can cause pathological disorders. Therefore, it is essential to reduce the inflammation, reducing the classical activated macrophages and, consequently, reduce the pro-inflammatory cytokines. In order to accomplish this, type II cytokines, like IL-4, IL-10 and IL-13 have to be secreted, so they can induce alternatively activated macrophages. These macrophages are involved in a longer survival of the host because they are more anti-inflammatory (Namangala *et al.* 2001). Thus, to facilitate the control of the parasitemia and the pathology, it is needed a shift from a type I inflammatory response, in an early stage, to a type II inflammatory response, in a later stage (Namangala *et al.* 2009).

Another mediator responsible, not just, for the control of the parasite levels, but also for the development of the pathology, is TNF-α (Magez *et al.* 1999). During trypanosome infections, TNF is involved both in parasitemia control and infection associated pathology such as anaemia, neurological disorders, fever and cachexia during both human and animal trypanosomiasis (reviewed in Taylor & Mertens 1999). VSG was identified as major TNF inducing component in trypanosome-soluble extract (Magez *et al.* 1998). In addition, protection from neuroinflammatory pathology of sleeping sickness has been associated with the levels of IL-10 and IL-6 in the brain (Sternberg *et al.* 2005).

Since this parasite is extracellular there is a dominant humoral response of the host. The activation of polyclonal B cells results in an increase in the number of B cells and an elevation in plasma immunoglobulin, mainly IgM (Hudson *et al.* 1976; Diffley 1983). The successive parasitemia waves are a result of the specific antibodies directed against the epitopes of the VSG coat. These antibodies are able to opsonize the parasites making possible the efficient phagocytosis and destruction of the immune complexes, in the liver, by the macrophages (Shi *et al.* 2004; Pan *et al.* 2006).

Trypanosome infections are characterized by a drastic suppression of the immune responses, which makes the host more susceptible to opportunistic infections. During the AT/HAT infections there is an inhibition of the T cell proliferation due to down regulation of IL-2 production and expression of IL-2 receptor, which leads to the host immunosuppression (Sileghem *et al.* 1989; Darji *et al.* 1992). It is believed, that TNF also plays a role in the process of suppressing the immune system (Schleifer & Mansfield 1993).

1.4 DNA vaccination

Since the first description where it was shown for the first time that an intramuscular injection of a plasmid was able to induce protein expression in muscle cells (Wolff *et al.* 1990), DNA vaccines have been object of many interest.

Followed this initial report on DNA vaccines there was a proliferation of studies from cancer (Buchan *et al.* 2005), to infectious virus diseases, most prominently HIV (Human Immunodeficiency Virus) (Hammer *et al.* 2013), hepatitis C virus (Frelin *et al.* 2004) and cytomegalovirus (Kharfan-Dabaja *et al.* 2013), but also parasitic diseases are potential targets to DNA vaccination, like malaria (Wang *et al.* 1998), Chagas disease (Quijano-Hernández *et al.* 2013) and African trypanosomiasis (Silva *et al.* 2009; Lança *et al.* 2011).

In order to create the plasmid, first the gene sequence of interest has to be generated, synthetically or by PCR, and then is enzymatically inserted into the multiple cloning region of the plasmid backbone, purified and delivered to the inoculation site. DNA vaccination is capable of inducing both, humoral and cellular immune responses (Figure 5). It starts when the optimized gene sequence is delivered, and the plasmid enters the nucleus of transfected local cells, such as myocytes (1) and antigen presenting cells (APCs) (2). Once this entry is completed, foreign antigens are generated as proteins, as a result of the expression of the plasmid-gene. These antigens become the subject of immune surveillance by the Major Histocompatibility Complex (MHC) class-I and -II molecules. APCs are able to present vaccine-derived endogenous peptides on

MHC-I molecules, which is followed either by direct transfection by the plasmid vaccine (2) or by cross-presentation of cell-associated exogenous antigens (3). Additionally, after the secretion of protein antigens that have been shed from transfected cells are captured and processed within the endocytic pathway, APCs mediate the display of peptides on MHC II molecules (4). Antigen-loaded APCs travel, via the afferent lymphatic vessel (5), to the draining lymph node, where they present antigenic peptide to naïve T cells, providing the necessary secondary signals to initiate an immune response and expansion of T cells (6), or instead, activation of B cells and antibody production (7). This activation is due to cytokines secreted by the CD4 T helper cells during cell-to-cell interaction with B cells, which is a response to peptide-bound MHC molecules and co-stimulatory secondary signals. Once T cells are primed in the draining lymph node they can be restimulated and expanded, by the presentation of the peptide-MHC complexes, at the immunization site. The activation of both T and B cells, elicit specific immunity against the antigen encoded by the plasmid. These cells travel through the efferent lymphatic system and provide a surveillance system (8) (reviewed in Kutzler & Weiner 2008).

Over the years there were some safety concerns about DNA vaccination: the potential to be integrated into cellular DNA, the development of autoimmunity and the possibility of antibiotic resistance. However, studies conform the safety of this class of vaccines. DNA vaccines tested did not shown relevant levels of integration into host cellular DNA, it typically occurs at lower rates than spontaneous mutation frequencies (Temin 1998; Manam *et al.* 2000; Ledwith *et al.* 2000). Considering the possible development of autoimmunity induced by DNA vaccines, studies made did not detect convincing evidence of autoimmunity (Le *et al.* 2000). With regards to antibiotic resistance, it is improbable that resistances emerge because the resistance genes contained in the plasmids are not commonly used to treat human infections. Although all of these concerns, DNA vaccination has been proven to be safe. And in order to respond to the concerns, the European Medicines Agency (EMA) and the US Food and Drug Administration developed guidelines on safety and testing DNA vaccines (Robertson & Cichutek 2000; Anon 2007).

In addition to this advantage, DNA vaccines are able to encode several types of genes and proteins, they are stable, ease to store and can be manufactured on a large scale (reviewed in Kutzler & Weiner 2008).

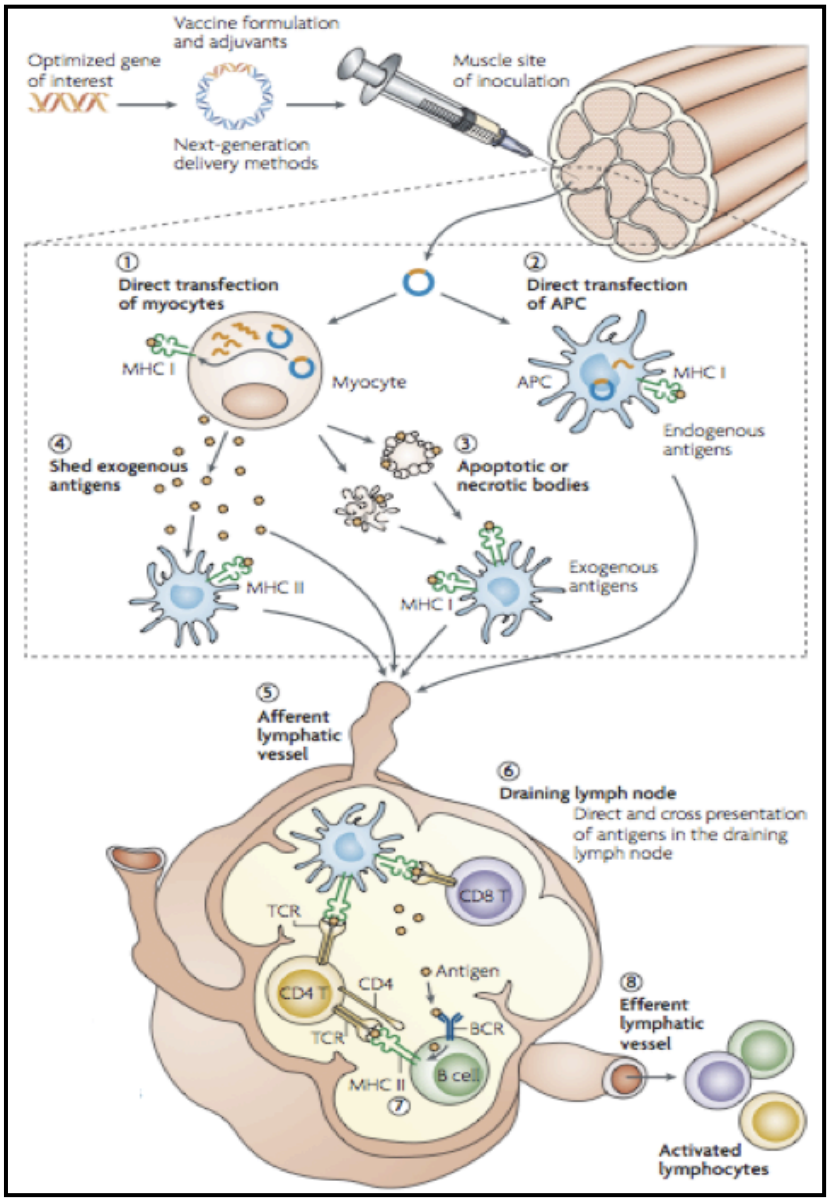

Figure 5: Schematic representation of the mechanism of action of DNA vaccines (Kutzler & Weiner 2008).

One of the disadvantages of DNA vaccination is the poor immunogenicity. In order to solve this problem, immunization techniques, nowadays, are changing and being improved. Electroporation is considered a promise as a second generation DNA vaccine technology for humans (Otten *et al.* 2004). This method consists on the application of an electrical pulse to the target tissue which disturbs membranes, leading

this way to an increase of cell permeability (Satkauskas *et al.* 2005). This approach expands DNA distribution in the injected muscles and uptake of DNA by myocytes which results in a higher efficiency of DNA vaccines (Dupuis *et al.* 2000), leading to an increase in the immune response and in the levels of expression (Hirao *et al.* 2008; Rosati *et al.* 2008; Vandermeulen *et al.* 2014). Other technique is the particle-mediated epidermal delivery, or particle bombardment, a needle-free DNA delivery technology that was developed as a physical gene transfer method to deliver DNA vaccines coated onto 1-3 μm gold beads into skin cells (reviewed in Yager *et al.* 2009; Wang & Lu 2013). Unlike routine intramuscular injection, this technique does not need larger amounts of DNA to have the same, or even higher, cellular and humoral immune response (Deng *et al.* 2014). This, may be due to the fact that DNA is delivered directly into the host's skin cells instead of in extracellular spaces (Roy *et al.* 2000). Another needle-free technique is high-pressure mediated delivery, it is similar to particle bombardment, forces a liquid through a small orifice under pressure to deliver the vaccine parentally (Mumper 2003; Graham *et al.* 2013). This technique, showed a higher immune response, when compared with approaches using needles or syringes (Graham *et al.* 2013), and it is being used in clinical trials of DNA vaccines to Ebolavirus and Marburgvirus, with positive results (Sarwar *et al.* 2014).

One example of a noninvasive technique is a dermal patch, like DermaVir (Genetic Immunity). It has the vaccine antigens encoded in a plasmid DNA formulated into a nanoparticle that target specialized dendritic cells under the skin. A single DermaVir immunization is able to produce a specific immune response (Cristillo *et al.* 2007; Jin & Kim 2014).

1.5 The Virus-like particles

Virus-like particles (VLP) are multimeric, sometimes multiprotein, supramolecular assemblages, geometrically well-defined, with diameters in the range of 25-100 nm, lacking genetic material, that mimic the overall structure of the native virions. The fact that recombinant VLPs do not contain viral genetic material, offers a safe method for prophylactic vaccination (reviewed in Chroboczek *et al.* 2014). The

idea of using non-infectious virus particles to develop prophylactic human vaccines has been attractive, since the discovery of non-infectious hepatitis B virus (HBV) particles (Millman *et al.* 1969).

Commonly, VLPs are more immunogenic than subunit or recombinant vaccines, and are able to stimulate the humoral and cellular immune system. VLPs have a multivalent display and a highly ordered structure that constitute the pathogen-associated molecular pattern motifs (PAMPs). Since these motifs are, by and large, unique to microbial antigens, the mammalian immune system has evolved to respond vigorously to this arrangement of antigens. PAMPs are able to trigger the innate immune sensing mechanisms and can be recognized by Toll-like receptors (TLRs) (reviewed in Plummer & Manchester 2011). VLPs are able to induce a strong B-cell response by efficiently cross-linking the membrane associated immunoglobulin molecules that constitute the B-cell receptor, thanks to the correct display of the antigenic epitopes and the highly repetitive structure (reviewed in Grgacic & Anderson 2006). VLPs are efficiently taken up by dendritic cells (DCs) for processing and presentation by MHC-II and for promoting DC maturation and migration, stimulating the innate immune system (Fifis *et al.* 2004; Gamvrellis *et al.* 2004). As native virus, VLPs can also be processed in the cytosol of DCs and are presented by MHC-I molecules to cytotoxic $CD8^+$ T cells by the cross-presentation mechanism, which is essential for the clearance of intracellular pathogens and for the induction of a potent cytotoxic immune response (Murata *et al.* 2003; Win *et al.* 2011).

Several VLP-based vaccines are already licensed. The first recombinant vaccine based on VLPs was the RECOMBIVAX HB® (Merck), against HBV (Krugman 1982). The next recombinant VLP-based vaccine took 20 years to be licensed: Gardasil® (Merck) against human papillomavirus (HPV). According to a study by the US Centre for Disease Control and Prevention, thanks to this vaccine there was a reduction of over 50% in HPV infection among teenagers (Markowitz *et al.* 2013). A second VLP-based HPV vaccine, Cervarix® (GlaxoSmithKline), was licensed in the USA, almost 3 years after Gardasil® (Deschuyteneer *et al.* 2014).

Based on the structure of their parental viruses, VLPs can be divided in two categories: non-enveloped and enveloped. The non-enveloped VLPs, such as the

licensed HPV VLP vaccines, consist of one or more components of a vaccine target antigens displayed on the VLP surface as a fusion to a heterologous viral protein with the ability to self-assemble, and they do not include any host components. These VLPs can be produced in prokaryotic and eukaryotic cells. On the contrary, the enveloped VLPs consist in the host cell membrane with the integrated target antigens displayed in the outer surface (reviewed in Lua *et al.* 2014).

VLPs are not just suitable as vaccines for homologous virus from which they are derived. Additionally, they can be used for the display of foreign antigens, in order to enhance their immunogenicity. Many soluble antigens have a poor immune response; this can be overcome by rendering them highly repetitive in a single particle. In this case, VLPs have two roles: they serve as scaffolds for presenting antigens derived from other pathogens in a suitable repetitive configuration, and as adjuvants to boost the immune response. An example of this kind of VLPs, is the new VLP-based vaccine for malaria, that includes a portion of the circumsporozoite protein of *Plasmodium falciparum* fused to the N-terminus of hepatitis B surface antigen (Rutgers *et al.* 1988) and is, already, in phase III of the clinical trial (Agnandji *et al.* 2012).

It was recently described a VLP based on the *Triatoma virus* (TrV) (Querido *et al.* 2013; Sánchez-Eugenia *et al.* 2015; Rodrígues Aguirre & Guérin 2015). This virus infects Triatomine bugs, the vector of *Trypanosoma cruzi*, the ethiological agent of Chagas Disease (Grayson 2010). The native TrV particles are composed by a 30 nm icosaedral capsid, with no lipid envelop. Its capsid is made up of 60 copies of a protomer, consisting of the structural proteins VP1 (29.7 kDa), VP2 (28.4 kDa) and VP3 (31.8 kDa), and VP4 (5.5 kDa) lying within the capsid and in contact with the RNA (Squires, Pous, Agirre, Rozas-Dennis, Costabel, G. a. Marti, *et al.* 2013).

2. Work Aims

2. Work Aims

The main aims of the present work are:

1- Cloning the genes MSP and PLC of the bloodstream forms of *Trypanosoma brucei brucei* in pVAX1 and pET28a;

2- Use the plasmids MSPpET28a and PLCpET28a to express the recombinant protein of MSP and PLC in *Escherichia coli*;

3- *In silico* analysis and design the antigenic epitopes as candidates to the VLPs.

4- Use the plasmids MSPpVAX1 and PLCpVAX1 as model to the development of DNA vaccines against *Trypanosoma brucei*, determine anti-*Trypanosoma brucei* antibodies and identify native proteins with serum from immunized mice by DNA vaccine, and challenge the mice with a lethal dose of *Trypanosoma brucei brucei*.

3. Materials and Methods

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3.1 Animals and parasites

All animals used in this work were obtained and maintained in the bioterium of the *Instituto de Higiene e Medicina Tropical*, Lisbon – Portugal. Female Balb/C mice, between 5 to 8 weeks old, were used in the experimental infection with *T. brucei* and in the DNA immunization protocols. The strain of *Trypanosoma brucei brucei* used in the experimental model was a GVR35 obtained from the *Unidade de Clínica Tropical* of the *IHMT*.

3.2 Infection protocol of mice and total protein extract from *Trypanosoma brucei brucei*

Mice were inoculated intraperitoneally with trypanosomes prepared by dilution of the frozen stabilate *T. b. brucei* GVR35 with PBS-glucose 20 mM pH 7.4. In order to obtain protein extract of *T. b. brucei*, blood from infected Balb/C was collected, by cardiac puncture, in syringes with heparin (B. Braun - Germany) as anti-coagulant. Then, 200 μL of PBS-glucose 20 mM pH 7.4, was added to the collected blood and submitted to purify bloodstream forms of the parasite using a gravity-operated DEAE-cellulose chromatography column, previously equilibrated with PBS-glucose 20 mM pH 7.4 (Lanham & Godfrey 1970). To evaluate the presence of bloodstream forms in the resultant fractions of the chromatography procedure, they were monitored by conventional optical microscopy. The fractions containing parasites were centrifuged, during 10 minutes at 380g, the pellet was suspended with PBS, and then frozen at -20ºC. When the samples were defrosted, a protease inhibitor was added. In order to determine the protein concentration of the extract, the *Bicinchoninic Acid Protein Assay Kit* (Sigma – USA) was used.

3.3 Obtaining genomic DNA from *Trypanosoma brucei*

The blood from the infected mice was used to purify genomic DNA from *T. brucei*, using a commercial kit *QIAamp® DNA Blood Mini Kit* (Qiagen – USA). After this step, the DNA concentration was determined by spectrophotometry at a wavelength of 260 nm. The DNA samples were used as template for the amplification assays of *Trypanosoma brucei brucei* genes of interest.

3.4 Obtaining the gene sequences of *Trypanosoma brucei*

Two *T. brucei* genes of interest were used as possible antigenic targets for the construction of DNA vaccine prototypes. The nucleotide sequences of these genes, named Major Surface Protease (MSP) and Phospholipase C (PLC), were obtained from GenBank® from National Library of Medicine – National Center for Biotechnology Information (NCBI), Bethesda – USA, under the gene identification (GI) AY230807 and XM_946646.1, respectively.

3.5 Construction and acquisition of the primers used in the amplification of *Trypanosoma brucei* genes of interest

To each gene of interest (MSP and PLC) a pair of primers were design to the terminal regions 5' and 3'. Subsequent to this, these primers, commercially acquired from Thermo Electron Corporation (Germany), were used in the amplification assays with Polymerase Chain Reaction (PCR).

As cloning strategy, was added to the extremities of each pair of primers the restriction sites to the enzymes NheI (GCTAGC) (Promega - USA) and BamHI (GGATCC) (Fermentas - Germany), used for the amplification of both, MSP and PLC

genes. The following table (Table 1) shows nucleotide sequences of the primers used in the PCR.

Table 1: Nucleotide sequences used in the primer synthesis for the gene amplification from *Trypanosoma brucei.*

Gene	Primer sequence
MSP 5'region (sense)	5'-GCTAGCATGACCCAACTGTTA-3'
MSP 3'region (antisense)	5'-GGATCCTCACATTTGACGAG-3'
PLC 5'region (sense)	5'-GCTAGCATGTTTGGTGGTGTA-3'
PLC 3'region (antisense)	5'-GGATCCTTATGACCTTGCGGT-3'

3.6 Polymerase Chain Reaction (PCR)

The PCR assays were used for the amplification of the gene of interest from *Trypanosoma brucei.* To this end, the amplification reaction was optimized under the conditions described below (Table 2):

Table 2: Mix constituents.

Constituents	Quantity
Genomic DNA from *Trypanosoma brucei brucei*	100 ng
Primers	100 pmol each
dNTPs	25 mM of each nucleotide dATP, dCTP, dTTP and dGTP)(Stratagene, California – USA)
$MgCl_2$ (Bioline – Germany)	3 mM
DNA Polimerase (BIOTAQ^{TM}, Bioline – Germany)	5 U
Reaction buffer NH_4 (Bioline – Germany)	5 µL
Final volume with sterile water	50 µL

After mixing the above constituents, the parameters used for the reaction were established in a thermocycler (Bio-Rad - USA): an amplification cycle with a denaturation temperature of 94ºC for 2 minutes, the annealing temperature of the primers of 55ºC for 1 minute and 30 seconds, and a extension temperature of 72ºC for 2 minutes, in a total of 40 amplification cycles.

After the amplification reaction, the samples were mixed with a Loading buffer (Bioline – Germany), and then submitted to a 0,8% (*w/v*) agarose gel electrophoresis (Bioline – Germany) with a Tris-Acetate-EDTA (TAE) with ethidium bromide (Sigma-USA).

The amplification products were then extracted from the gel and purified with a commercial kit, *QIAquick Gel Extration Kit* (Qiagen - USA). In order to determine the size of the amplified fragments, was used the molecular weight marker Hiperladder I^{TM} (Bioline – Germany).

3.7 Construction of DNA vaccine prototypes

3.7.1 Plasmid construction

All the vectors used in the immunization process were derived from the commercially available plasmid vector pVAX1 (2999 bp, Invitrogen - USA) (Figure 6), designed to be use in the development of DNA vaccines. This vector contains the human cytomegalovirus (CMV) immediate-early promoter, the bovine growth hormone (BGH) polyadenylation signal and a kanamycin resistance gene.

The genes amplified by PCR were submitted to a cloning strategy in the plasmid pVAX1. In order to accomplish this, both, fragments and the plasmid vector pVAX1, were submitted to a digestion step (37ºC for 3 hours) with restriction enzymes (NheI and BamHI), followed by a dephosphorylation step of the plasmid, using the enzyme alkaline phophatase (Promega - USA), with the purpose of removing the phosphate group in the 5’ region. The products from de enzymatic digestion, were, then, submitted

to a 0.8 % (*w/v*) agarose gel electrophoresis and the fragments were extracted from the gel and purified by *QIAquick Gel Extraction Kit* (Qiagen – USA). The products of this purification were cloned. To accomplish this, the enzyme DNA ligase (Promega - USA) was used, and the manufacturer's instructions were followed.

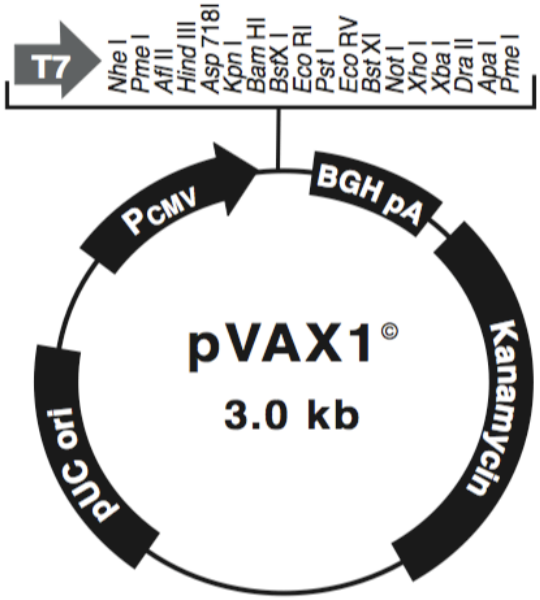

Figure 6: Schematic representation of the plasmid pVAX1 (Invitrogen 2002).

3.7.2 Bacterial transformation

Plasmid DNA was added to competent cells, *Escherichia coli* DH5α cells, and submitted to a heat shock. First, the mixture was kept on ice for 30 minutes, then heated at 42ºC during 1 minute, and, immediately after, cooled down on ice for 2 minutes. The mixture was placed in 1 mL of LB Broth (Sigma - USA) medium and incubated for 40 minutes to one hour at 37ºC. Then, the culture was centrifuged at 855g for 5 minutes, the resultant pellet was suspended and placed on LB/Agar (Sigma – USA) plates containing 30 µg/mL (*w/v*) kanamycin for selection of transformed *E. coli* colonies and was incubated overnight.

Plasmid DNA used in the immunization process was prepared from 250 mL of *E. coli* DH5α in 20 g/L of LB medium with 30 µg/mL (*w/v*) of kanamycin (Bioline - UK), overnight at 37ºC in an orbital shaker.

3.7.3 Plasmid Purification

Plasmid purification was performed by two different techniques, taking into account the quantity of plasmid needed: by standard alkaline lysis followed by hydrophobic interaction chromatography (HIC) (Diogo *et al.* 2000), when higher amounts of plasmid were needed, for example for the immunizations; and when lesser amounts were needed, was used the *ISOLATE II Plasmid Mini Kit* (Bioline - UK).

Initially, to perform the alkaline lysis, the inoculum was centrifuge at 3000g for 15 minutes at 5ºC. After discard the supernatant, was added 8 mL of solution P1 (in section 7), 8 mL of solution P2 (in section 7) and incubated for 5 minutes at room temperature, and then 8 mL of solution P3 (in section 7) and incubated for 5 minutes in ice. After all the solutions were added, the mixture was centrifuged for 30 minutes, at 18000g. The supernatant was transferred to a new tube, and centrifuged one more time, in order to remove all the floating material. The clean supernatant, was then passed to a new tube and was added 0.8 of the final volume of isopropanol (Panreac – Germany), and cooled down at 4ºC overnight.

The hydrophobic interaction chromatography, started with a centrifugation at 18000g, during 30 minutes, at 5ºC. After the centrifugation, the pellet was washed with 2 mL of ethanol at 70%, in a way, that just the DNA stays undissolved. Another step of centrifugation, in the previous conditions was performed, and the supernatant was discarded and the pellet was left to dry. Then it was suspended in 500 µL of Solution 1 (in section 7) and placed in microtubes of 1.5 ml with 0.16 g of ammonium sulphate, and centrifuged at 9503g for 5 minutes. The 500 µL of sample were placed in the column, previously equilibrated with Solution 2 (in section 7). After the column stopped dripping, first, 3 mL of Solution 2 was added, and then 2 mL. So, the final volume of this step was 2 mL.

In order to remove the salt from the HIC step, a gel-filtration was performed. The resulting 2 mL were added to a second column, previously equilibrated with PBS 1X. Then, 1.5 mL of PBS 1X was added, to bring down the plasmid, and finally, 3 mL of the same solution to recover the plasmid. When the plasmid was recovered, the same volume of isopropanol was added and left overnight at 4ºC. The last step of the purification consists of a centrifugation for 30 minutes, at 18000g. The supernatant was discarded and the pellet suspended in 1 mL of PBS 1X. The optical density of the purified plasmids was measured at 260 nm. To confirm the prevalence of the super coiled form of the plasmid, the samples were submitted to a 1% (*w/v*) agarose gel electrophoresis. The plasmids used in the immunization process were characterized by automated sequencing (STABVIDA, Lisbon – Portugal) and by a restriction analysis.

3.8 Subcloning the genes into plasmid pET28a

In order to express the recombinant proteins MSP and PLC in *E. coli*, was used the pET28a plasmid vector (5369 bp, Novagen - Germany) (Figure 7), designed to be use in the expression of recombinant proteins in *Escherichia coli*. So, to achieve this, it was needed a subcloning step. The MSPpVAX1 and PLCpVAX1 plasmids were digested (37ºC for 3 hours) with the restriction enzymes NheI and BamHI. The pET28a plasmid was also submitted to a double digestion in the same conditions.

As soon as the digestion is completed the products were submitted to a 0.8% (*w/v*) agarose gel electrophoresis and the fragments with the genes were extracted from the gel and purified with the *QIAquick Gel Extraction Kit* (Qiagen – USA). The sub-cloning process was accomplished as described in the section 3.7. The sub-cloned plasmids were characterized by automated sequencing (SECUGEN - Madrid) and by a restriction analysis with NheI and NotI (Promega - USA), at 37ºC for 3 hours.

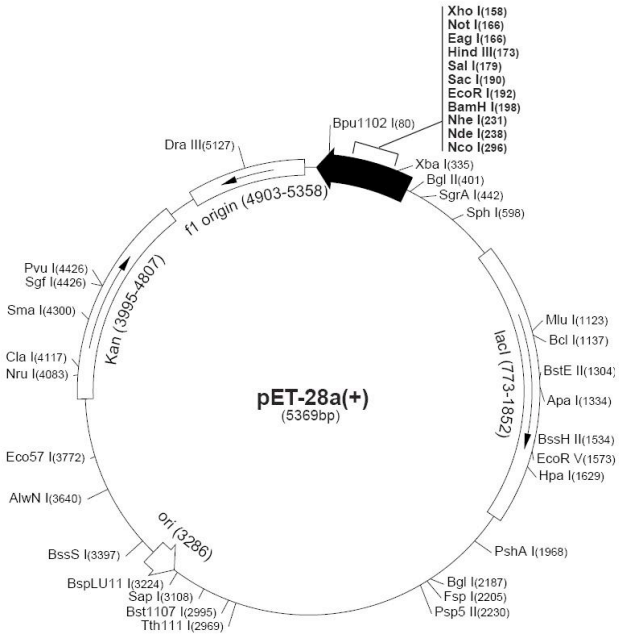

Figure 7: Schematic representation of the plasmid pET28a (Novagen 2003).

3.9 Immunization Protocol

The Balb/C mice were divided in 5 groups, with 3 animals per group, and each was immunized subcutaneously with 100 μg of the vaccine candidates, Table 3. There was a control group that was injected with the same amount of pVAX1. In the groups 1 to 4, the plasmids were eluted in PBS 1X pH 7.4, and the group 5, in a solution of 10 mg/mL of nanoxylan. This is a nanoformulation that is derived from brown seaweed: *Spatoglossum schröederi*. It has been proven to have antiproliferative, anti-adhesive, antiangiogenic and antithrombotic properties (Dantas-Santos *et al.* 2012; Negreiros 2015).

Table 3: Vaccine composition per group.

Groups	Vaccine composition
G1	pVAX1
G2	MSPpVAX1
G3	PLCpVAX1
G4	MSPpVAX1 + PLCpVAX1
G5	MSPpVAX1 + PLCpVAX1

It was used three immunization steps and six blood collection, as demonstrated in the following figure.

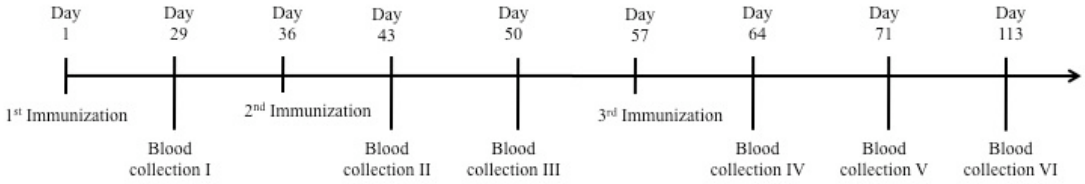

Figure 8: Immunization protocol.

3.10 Harvesting the serum of the immunized mice

The blood samples of the immunized mice were collected at 6 different time points. The blood was collected through terminal tail.

After the blood was collected, it was placed at 37ºC for 30 minutes and then at 4ºC for 1 hour. To obtain the serum, the samples were centrifuged at 3421g for 10 minutes, and, finally, the serum was stored at -20ºC.

3.11 Determination of anti-*Trypanosoma brucei* antibodies by ELISA in mice immunized by DNA vaccine

Total IgG antibodies anti-*T. b. brucei* were titled by Enzyme-Linked Immunosorbent Assay (ELISA), using serum samples from immunized mice. First, 96-well microplates (Costar - USA) were coated with 100 ng/well of total protein extract from *Trypanosoma brucei brucei* dissolved in bicarbonate buffer pH 8.5 (in section 7), overnight in moist atmosphere at 4ºC. The microplates were, then, washed three times with 0.05% Tween 20 (Sigma-Aldrich - USA) in PBS 1X and blocked for one hour with 2% casein (*w/v*) (Sigma – USA) in PBS 1X, at room temperature. After three wash-ups, serial dilution of serum from immunized mice in antibody buffer (0,4% casein in PBS 1X) added to the plates and incubated for one hour at room temperature. The microplates were washed five times and, then incubated, for 1 hour, with horseradish peroxidase (HRP)-conjugated antibodie: α-mouse IgG (Sigma - USA) diluted 1:4000. After another step of five wash-ups, it was added the substrate solution: 10 mL of citrate buffer pH 5.0 (in section 7), 10 mg of OPD (o-phenylenediamine dihydrochloride) (Sigma - USA) and 10 µL of H_2O_2 30 % (*v/v*) (Sigma-Aldrich - USA), for 30 minutes at room temperature and in the dark. To stop the reaction, after the 30 minutes, 50 µL/well of 4N sulphuric acid was added, and the optical density was measured at 490 nm.

3.12 Expression of the recombinant MSP and PLC proteins in *Escherichia coli*

Cells BL21 (DE3) (Novagen – Germany), BL21 (DE3) pLys (Novagen – Germany), Rosetta (DE3) (Novagen – Germany) and Origami2 (DE3) pLys (Novagen – Germany) from *E. coli*, were transfected with MSPpET28a and PLCpET28a and inoculated with LB medium at 37ºC and with agitation, until reaching, approximately, 0.8-1 optical density (600 nm). An aliquot of 200 µL was, then, placed in a new LB medium and the expression of the respective recombinant proteins were induced by adding 5 µL of 0.5 mM IPTG (Bioline – UK) in different time-points and at different temperatures, in agitation. The remaining non-induced cells were used as negative

control. The cells were centrifuged at 6000g for 10 minutes at 4ºC, and the pellet was suspended in PBS 1X pH 7.4. Subsequently, SDS-sample buffer 1:1 (*v/v*) was added to the samples, and they were reduced by putting them at 100ºC for 7 minutes. After the reduction step, the samples were submitted to a sodium dodecyl sulfate 10% (*w/v*) polyacrylamide gel electrophoresis (SDS-PAGE) and then stained with Comassie Brilliant Blue.

3.13 Immunological identification of native MSP and PLC proteins of bloodstream forms of *T. b. brucei* with serum from immunized mice

In order to confirm the presence of the proteins used in the immunization protocol, the Western Blot technique was used.

The total protein extract of *Trypanosoma brucei brucei* was submitted to a SDS-PAGE.

The gels were blotted onto nitrocellulose membranes (Whatman Protra - Germany) by electric transference at 4ºC in blotting buffer, overnight. The membranes were washed two times, for 10 minutes, with a washing buffer (0.05% Tween 20 in TBS 1X) and then blocked with a blocking buffer (3% powder milk (*w/v*) in TBS 1X) for 1 hour at room temperature. Afterward, the membranes were washed five times, for 1-2 minutes, and incubated with the serum from the immunized mice diluted in a antibody buffer (0.1% powder milk (*w/v*) in TBS 1X) 1/10 (*v/v*) for 1 hour at room temperature on orbital shaking. The membranes were washed and incubated, for 1 hour, with horseradish peroxidase (HRP)-conjugated antibodie: α-mouse IgG diluted 1:4000. After this, the membranes were washed five times with washing buffer, in orbital shaking. The development solution, DAB (3,3' – Diaminobenzidine tablets – SigmaFast, Sigma-Aldrich - USA) 0.5 mg/mL in TBS 1X with 0.02% H_2O_2, was added and incubated at room temperature until the color development (2-10 minutes). The reaction was stopped with deionized water and the membrane was left to dry.

3.14 Parasite challenge of the immunized mice

The previously immunized mice were infected with 500 parasites/animal of the infecting form of *Trypanossoma brucei brucei*, by intraperitoneal injection. The infection took place 92 days after the last immunization. The survival of the infected animals was registered daily. The number of parasites in the blood was monitored on days 5, 8, 10, 12 and 15 post-infection, by smear stained by Giemsa. The method used to count the parasites was adapted from Luz *et al.* 2005: were counted 25 fields per slide and then was made an estimate to 100 fields.

3.15 Analysis *in silico* of MSP and PLC genes

With the resulting sequences from automated sequencing, a brief analysis *in silico* was made, to collect some information about the proteins being used.

In order to achieve this, the resulting sequences were treated with the software Geneious® (version 8.1.2) (Kearse *et al.* 2012). Then, a BLAST was made, with this step was obtained the protein sequence. Using ProtParam (Gasteiger *et al.* 2005) from Expasy, it was possible to obtain the molecular weight and the theoretical pI, which is needed to separate the proteins in a polyacrylamide gel using isoelectric focusing. As mentioned before, section 1.2.4, both proteins are anchored to GPI, so the tool GPI-Predictor (Eisenhaber *et al.* 1998; Sunyaev *et al.* 1999; Eisenhaber *et al.* 1999; Eisenhaber *et al.* 2000) was used, in order to verify if this happened with the proteins used here. Ultimately, with TMHMM 2.0 (Sonnhammer *et al.* 1998; Krogh *et al.* 2001), was possible to determine where the proteins were located in the cell.

3.16 Design of the antigenic epitopes as candidates to the VLPs

The process of designing the antigenic epitopes, using the MSP and PLC from *T. b. brucei*, as candidates to the VLPs was a four-step process. In this process, was used the crystallographic structure of TrV (*Triatoma Virus*) (Squires *et al.* 2013).

The first step consisted of bibliographic research, in order to obtain as much information as possible about the proteins used, like, the tertiary structure and existence of epitopes. The tertiary structure was searched in the Protein Data Bank (Berman *et al*. 2000) using the keywords: "Major Surface Protease", "MSP", "*Trypanosoma brucei*", “PLC” and “Phospholipase-C”.

In the second step, the insertion region of the epitopes was selected from the atomic structure of the TrV. To complete this step, first an analysis of the tertiary structure, by the PyMOL software (Schrodinger LLC 2010), was made, in order to see where the epitopes could be located in the protein and a qualitative analysis of the exposure to the solvent of the epitopes. The ASA-View (Ahmad *et al.* 2004) made the quantitative analysis of this exposure. The exposure is a residue characteristic that measures the accessibility to the solvent. The epitopes should be exposed in order to be recognized by the immune system (Joshi *et al.* 2013).

The third step of the process was the construction of the atomic model of the chimerical particle, using a software called Coot (Emsley & Cowtan 2004). In this step, the amino acids from the native TrV structure were changed by the amino acids of the epitopes.

The fourth step consisted of determining how the changes made by the insertion of the epitopes into the TrV native structure changed both the epitopes and the TrV properties. In order to accomplish this, all the structures (protein, native TrV and mutated TrV) were carefully analyzed and compared between each other. There were three criteria compared: exposure, salt bridges and disulphide bond. The salt bridges were analyzed by ESBRI (Costantini *et al.* 2008) and VMD (Humphrey *et al.* 1996); and to analyze the disulphide bonds was used a script from www.pymolwiki.org.

An additional software, UCSF Chimera (Pettersen *et al.* 2004), was used in order to obtain detailed images of the epitopes.

4. Results and Discussion

4. Results and Discussion

4.1 Restriction Analysis of the plasmids

4.1.1 Restriction Analysis of the plasmids cloned in pVAX1

The previously cloned plasmids MSPpVAX1 and PLCpVAX1 were submitted to a restriction analysis with NheI and BamHI, as explained in section 3.7.3, and analyzed by a 1% (*w/v*) agarose gel electrophoresis. The Figure 9 shows the results from the restriction analysis of the cloned plasmids.

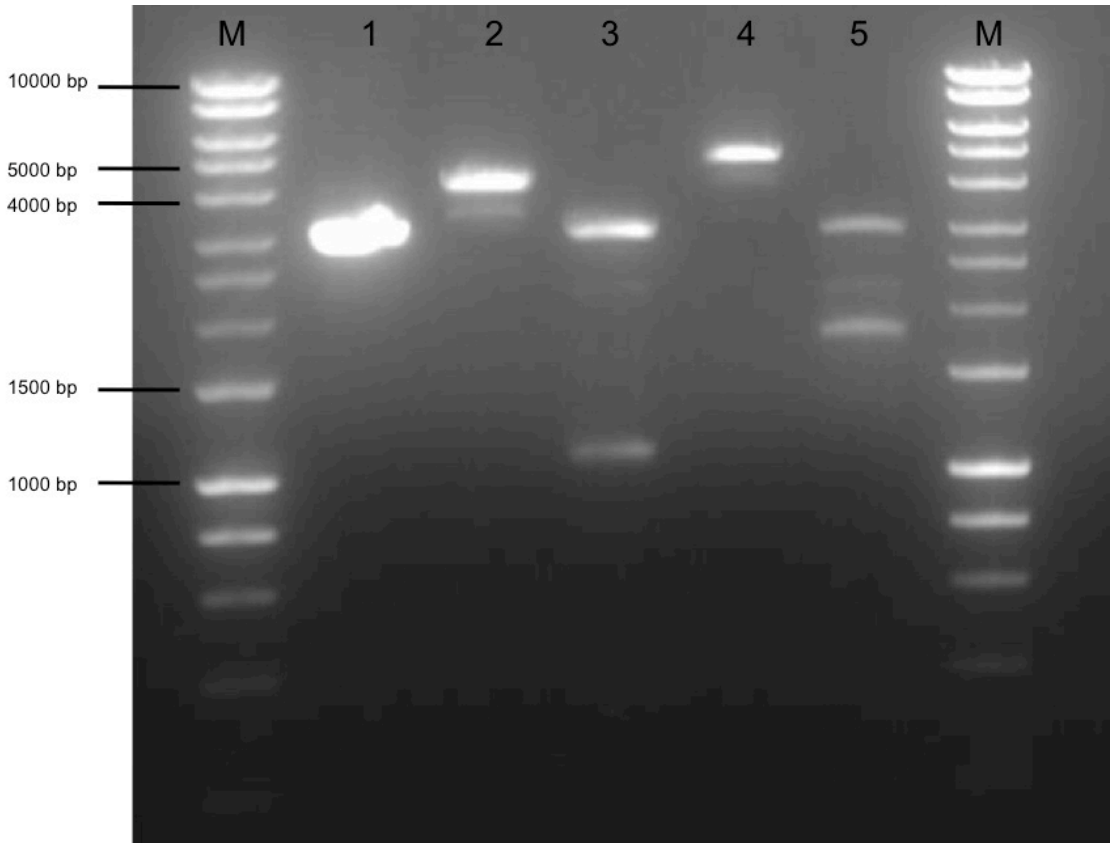

Figure 9: Restriction analysis of the genes MSP and PLC cloned in pVAX1. **M-** Marker HyperLadder™ I (Bioline – UK); **1-** pVAX1; **2-** PLCpVAX1 digested with BamHI; **3-** PLCpVAX1 digested with NheI and BamHI; **4-** MSPpVAX1 digested with BamHI; **5-** MSPpVAX1 digested with NheI and BamHI.

In the well **1** it is shown the plasmid pVAX1 (≈ 3.0 kb) without any gene or enzyme restriction.

The restriction of the PLCpVAX1 suggests the cloning of the fragment PLC (≈ 1.1 kb) into the pVAX1 plasmid, generating a fragment of approximately 4.1 kb when digested with BamHI (well **2**). This suggestion is reinforced by the restriction analysis with NheI and BamHI enzymes (well **3**), because it shows two fragments: the first one of ≈ 3.0 kb (pVAX1) and ≈ 1.1 kb (PLC).

When the plasmid MSPpVAX1 was digested with BamHI (well **4**) a fragment of approximately 4.7 kb was present, suggesting the insertion of the fragment MSP (≈ 1.7 kb) into the pVAX1 plasmid (≈ 3.0 kb). When the MSPpVAX1 was digested with BamHI and NheI (well **5**), two fragments appeared: one of ≈ 3.0 kb, that corresponds to **pVAX1** and another of ≈ 1.7 kb that corresponds to MSP.

After the restriction analysis, both plasmids (MSPpVAX1 and PLCpVAX1) were submitted to an automated sequencing to determine the gene sequence and to compare them with the original sequences.

4.1.2 Restriction Analysis of the plasmids sub-cloned in pET28a

The plasmids MSPpVAX1 and PLCpVAX1 were submitted to a sub-cloning step: the genes MSP and PLC were sub-cloned into the pET28a plasmid, as referred in section 3.8. After this, both plasmids were submitted to a restriction analysis, seen in Figure 10. The main aim of this sub-cloning step was to express the recombinant proteins MSP and PLC, in prokaryotic cells.

When the plasmid MSPpET28a was digested with HindIII (well **1**) a fragment of ≈ 7.0 kb was present, suggesting the insertion of the fragment MSP (≈ 1.7 kb) into the plasmid pET28a (≈ 5.3 kb). This is supported by the restriction analysis with NheI and NotI enzymes (well **3**), which shows the presence of two fragments: the first one, of ≈ 5.3 kb, that corresponds to pET28a; and the second, of ≈ 1.7 kb, corresponding to the fragment MSP.

The restriction analysis of the PLCpET28a also suggests the cloning of the fragment PLC (≈ 1.1 kb) into the pET28a plasmid, which generated a fragment of approximately 6.4 kb when digested with HindIII (well **2**) and two fragments of ≈ 5.3 kb and ≈ 1.1 kb (well **4**), corresponding to pET28a and PLC, respectively.

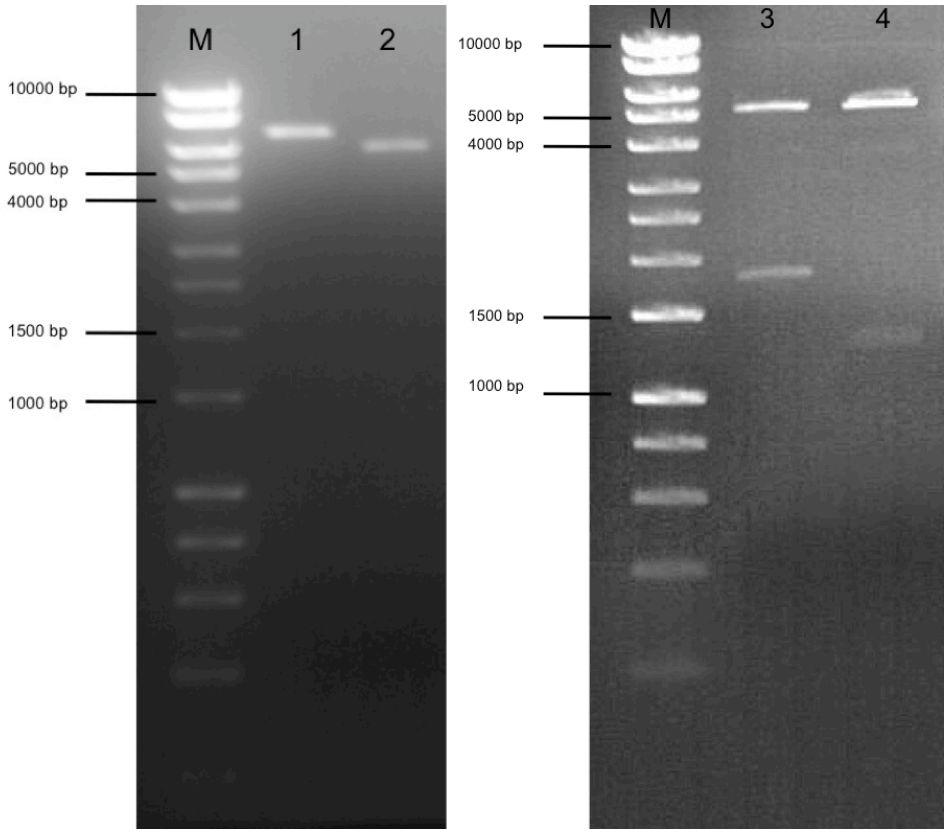

Figure 10: Restriction analysis of the genes MSP and PLC cloned in pET28a. **M-** Marker HyperLadder™ I (Bioline – UK); **1-** MSPpET28a digested with HindIII; **2-** PLCpET28a digested with HindIII; **3-** MSPpVAX1 digested with NheI and NotI; **4-** PLCpVAX1 digested with NotI and NheI.

After the restriction analysis, both plasmids (MSPpET28a and PLCpET28a) were submitted to an automated sequencing to determine the gene sequence and to compare them with the original sequences (in Attachment A-E).

4.2 Analysis by automated sequencing of the MSP and PLC genes in pVAX1 and pET28a

After the restriction analysis, the plasmids were analyzed by automated sequencing to determine the nucleotide sequence from the MSP and PLC genes from *T. brucei*.

The results of the sequencing are shown bellow (Table 4), and show the identity between the genes cloned in both plasmids (pVAX1 and pET28a) and the original sequences of MSP and PLC from the GenBank®, AY230807 and XM_946646.1, respectively.

Table 4: Alignment of the sequencing results with the original sequence from the MSP and PLC genes from *Trypanosoma brucei brucei*.

Plasmid	Primer	Sequenced region	Identity (%)
MSPpVAX1	T7 fwd	1083 bp	99
MSPpVAX1	BGH rev	543 bp	99
PLCpVAX1	T7 fwd	1176 bp	99
PLCpVAX1	BGH rev	689 bp	99
MSPpET28a	T7p	1296 bp	99
MSPpET28a	T7t	1303 bp	99

The previous table does not show the results from automated sequencing of the plasmid PLCpET28a because they came back unclear and was impossible to analyze them.

Together, the results from the restriction analysis and the sequencing, confirm the cloning, the identification and the characterization of the plasmids constructed in pVAX1, which were then used to sub-clone in pET28a plasmids.

4.3 Purification of the plasmids used in the immunization protocol

After cloning, identification and characterization, the MSPpVAX1 and PLCpVAX1 were produced in *Escherichia coli* DH5α cells, and then purified by hydrophobic interaction chromatography and gel filtration, according to Diogo *et al*. 2000.

In order to obtain a sufficient amount of plasmid to immunize all of the mice three times, several processes of production and purification were needed. In the end, a pool with all the produced and purified plasmid was made, and some laboratory parameters were observed (Table 5), such as concentration and purity of the plasmid, determined by optical density with a wavelength of 260 and 280 nm.

Table 5: Parameters of the plasmids used in the immunization protocol.

Plasmid	Concentration (µg/mL)	Ratio 260/280 nm
pVAX1	775	1.733
MSPpVAX1	600	1.282
PLCpVAX1	575	1.256

4.4 Determination of anti-*Trypanosoma brucei brucei* antibodies in mice immunized by DNA vaccine

As described, previously, in section 3.9, 15 Balb/C mice were divided in 5 groups of 3 mice per group. Each mouse was immunized three times intramuscularly with 100 µg of plasmid. There were six different time points of serum collection (day 29, 43, 50, 64, 71, 113 post-immunization), after each one of them the samples were tested by ELISA, in order to determine the anti-*Trypanosoma brucei brucei* antibodies production, therefore, the humoral response. A total protein extract from *T. brucei brucei* was used as an antigen source. The results of this assay, in figure 11, are presented as a relation between time of immunization and optical density.

The genes used to develop the vaccines (MSP and PLC) were selected due to the fact that both are expressed during the bloodstream forms of the parasite, they act synergistically to the loss of VSGs during differentiation from bloodstream form to procyclic form and consequently to the evasion of the immune system (Carrington *et al.* 1998; Grandgenett *et al.* 2007; Hanrahan *et al.* 2009). There are several works suggesting that the injection of plasmids encoding different proteins from *T. b. brucei*, specifically ISG and nTSA, results in humoral response and partial immune protection (Silva *et al.* 2009; Lança *et al.* 2011).

The results presented in Figure 11, confirm the production of IgG antibodies anti-*T. b. brucei*. The dilution of serum used in this assays was 1:100 (*v/v*). A pattern of response can be observed: after each immunization the optical density decreases, meaning there is an adaptation period. After this, the antigen becomes more immunogenic due to the activation of B-cells, resulting in an increase of optical density. Finally, because of the half-life period of the antibodies there is a decrease in the antibodies titre. A higher and faster response was observed after the second immunization, due to the presence of memory cells. However, the titre values should be higher than the control (G1), in order to be considered as humoral response. The groups where this characteristic is more evident are the ones immunized with PLCpVAX1 (G3) and with MSPpVAX1+PLCpVAX1+nanoxylan (G5).

Taking into account that the two groups immunized with both plasmids (G4: MSPpVAX1+PLCpVAX1 and G5: MSPpVAX1+PLCpVAX1+nanoxylan) have the same components and that the one with nanoxylan has a higher immune response than the one without it, this can mean that the nanoxylan works as an adjuvant.

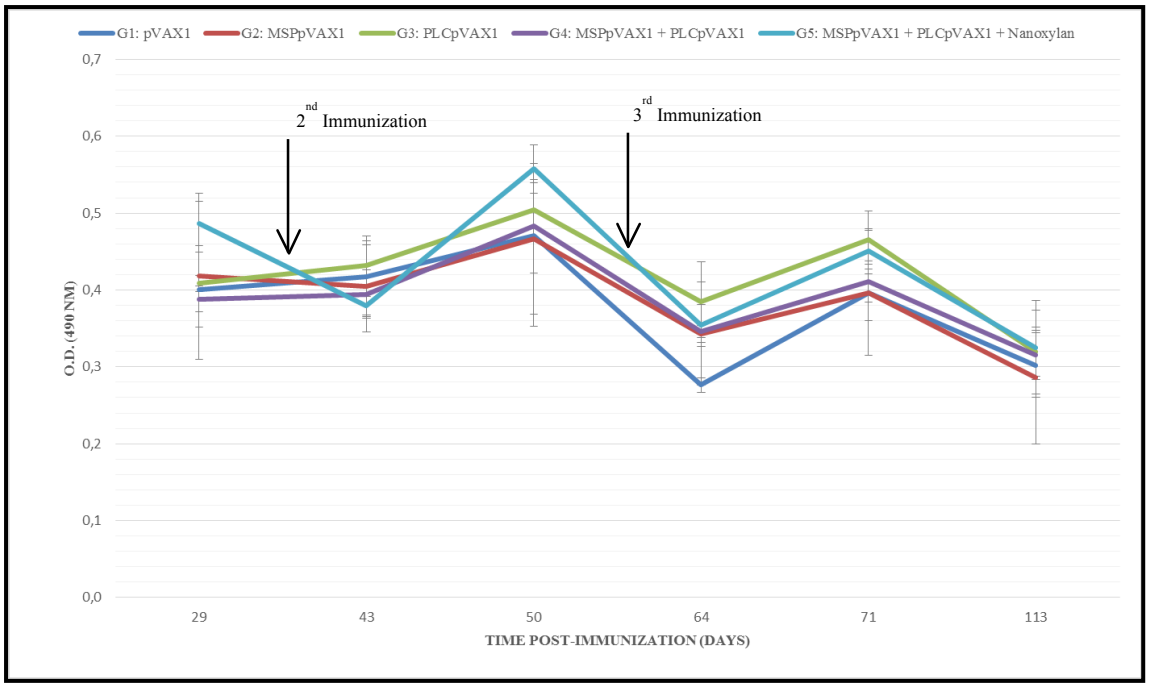

Figure 11: IgG anti-*Trypanosoma brucei brucei* humoral immune response elicited by DNA vaccination.

4.5 Immunological identification of native and recombinant *Trypanosoma brucei brucei* proteins from immunized mice

In order to identify immunologically recombinant MSP and PLC proteins, competent cells from *E. coli* had to be transfected with the plasmids PLCpET28a and MSPpET28a. The technique specified in section 3.12, was tried at different conditions: different temperatures (25ºC, 28ºC and 37ºC) and different times of induction with IPTG (0h, 1h, 2h, 3h and 4h); and then, submitted to SDS-PAGE and stained with Comassie Brilliant Blue. However, the proteins were not expressed in any of these conditions. Several works report the difficulty of producing membrane proteins due to their partially hydrophobic surfaces, flexibility and lack of stability. Moreover, it has been proven that the expression of functional eukaryotic membrane proteins in prokaryotic hosts is very difficult, due to a number of factors: the difference of lipid composition between bacterial and eukaryotic membranes, which could lead to an unreceptive environment; and incapacity of bacterial hosts to glycosylate or modify,

post-translationally, the eukaryotic proteins. Nowadays there is a large number of expression systems that have been proven to work better with eukaryotic membranes: yeasts, insect cells among others (reviewed in Loll 2003; Carpenter *et al.* 2008).

In the Figure 12, is represented one of the attempts to express the recombinant proteins, where the nTSA (wells 1-4) served as positive control. As can be seen, MSP (wells 5-8) and PLC (wells 9-12) were not expressed.

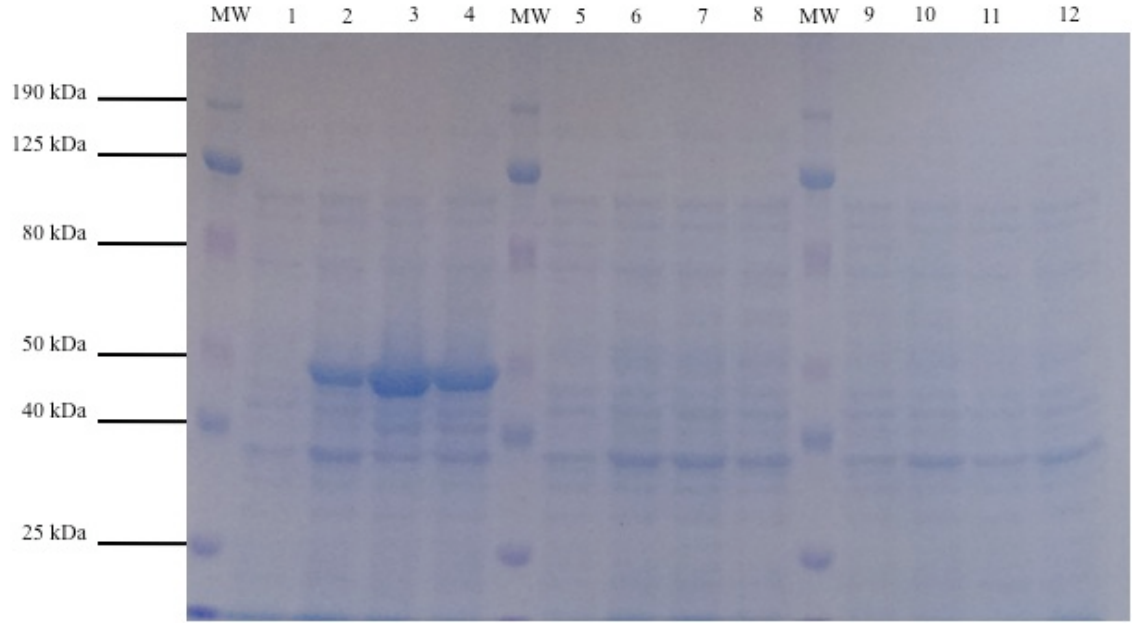

Figure 12: Results of the SDS-PAGE to test the expression of the recombinant proteins. **1-** Negative control of the expression of nTSA; **2-** Expression of nTSA induced with IPTG at 0h; **3-** Expression of nTSA induced with IPTG at 2h; **4-** Expression of nTSA induced with IPTG at 3h; **5-** Negative control of the expression of MSP; **6-** Expression of MSP induced with IPTG at 0h; **7-** Expression of MSP induced with IPTG at 2h; **8-** Expression of MSP induced with IPTG at 3h; **9-** Negative control of the expression of PLC; **10-** Expression of PLC induced with IPTG at 0h; **11-** Expression of PLC induced with IPTG at 2h; **12-** Expression of PLC induced with IPTG at 3h; **MW-** Molecular weight marker HyperPAGE® (Bioline – Germnay).

The next step was to identify native MSP and PLC proteins from serum from the immunized mice.

First, to select the optimal concentration of total protein extract for a better gel resolution and visualization of the proteins from *T. b. brucei*, this one was submitted to a SDS-PAGE with different amount of proteins extract (Figure 13). The amount of protein extract used was $10x10^3$ µg/well.

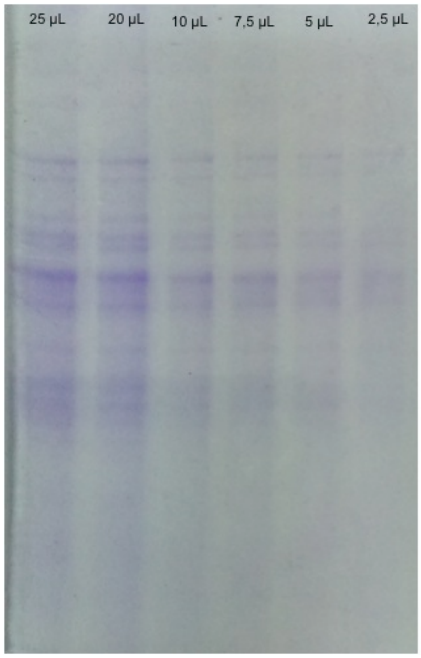

Figure 13: Electrophoretic profile of total protein extract of *Trypanosoma brucei brucei* in polyacrylamide gel electrophoresis and stained with Comassie Brilliant Blue. Samples 25µL to 2,5 µL/well: amount (in volume) of protein extract with 500 µg/µL.

After the optimal amount of protein extract had been selected the parasite extract was submitted to a Western Blot technique, in order to confirm the presence of the proteins being used in the immunization protocol. With all the serums collected from the immunized mice, a pool was made per group. The dilution of serum used in this assays was 1:10 (*v/v*).

According to the literature and the, analysis *in silico* (Section 4.7, Table 6), it was expected that the serum from immunized mice with MSPpVAX1 presented a band with ≈ 60 kDa (Grandgenett *et al.* 2007) and, in the case of, PLCpVAX1 the molecular weight was ≈ 40 kDa (Hereld *et al.* 1986; Carrington *et al*. 1998). As can be seen in Figure 14, the results were not the expected: the molecular weights do not correspond with the ones from the literature. The presence of bands in the control group (G1) could be a result of non-specific reactions (cross-reactions with non-specific antibodies of *Trypanosoma brucei brucei*). Another feature that is relevant to discuss, is the fact that, although, several dilution of the serum were tried, none of them resulted in bands with high intensity. This results together with the ones from the ELISA, suggest that the proteins are being expressed in low levels.

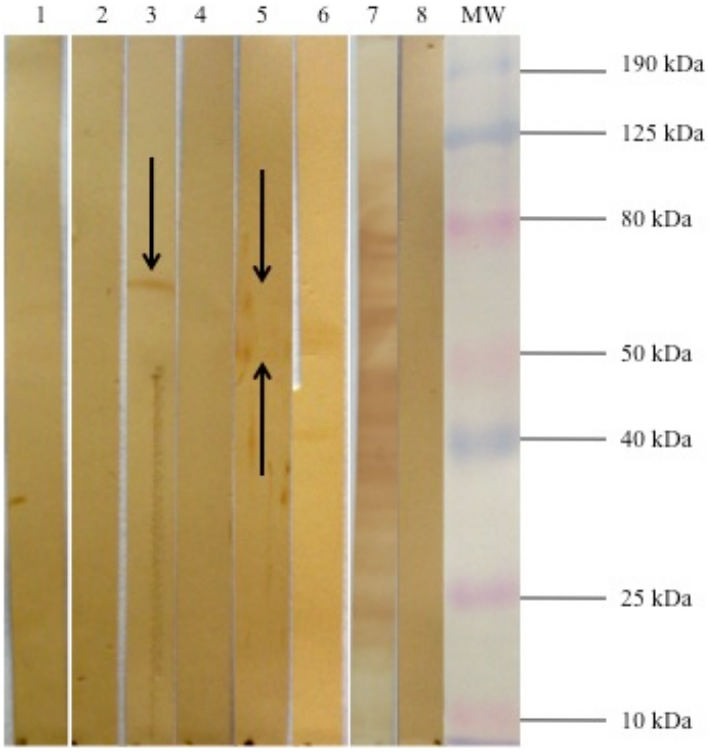

Figure 14: Immunodetection of antibodies in pools of serum of the immunized mice. **1-** G1: pVAX1; **2-** G2: MSPpVAX1; **3-** G3: PLCpVAX1; **4-** G4: MSPpVAX1+PLCpVAX1; **5-** G5: MSPpVAX1+PLCpVAX1+nanoxylan; **6-** Positive control (serum from an infected mice with 24 DPI)**; 7-** Polyclonal; **8-** Negative control; **MW-** Molecular weight marker HyperPAGE® (Bioline – Germnay).

4.6 Challenge assays of immunized mice

In order to test the efficiency of the vaccines, the previously immunized mice, were challenged with *Trypanosoma brucei brucei*. This assay consisted in the intraperitoneally injection of 500 parasites/animal, 149 days after the first immunization. After the infection, the animals were monitored daily, smears were peaked on days 5, 8, 10, 12 and 15 post-infection, and the survival rate was evaluated and compared between each group.

As can be seen in Figure 15, the infection protocol was successfully executed: parasites appear in circulation 5 days post-infection (DPI), and the pattern of infection is similar to the one presented in section 1.2.1 (Figure 3), distinctive feature of the *Trypanosoma brucei* infection. Although the differences between groups are not that evident, a small difference can be noticed: in G3 and G5, the parasitemia peak is lower than in the other groups; and G3 reaches the lowest level of parasitemia at 10 DPI.

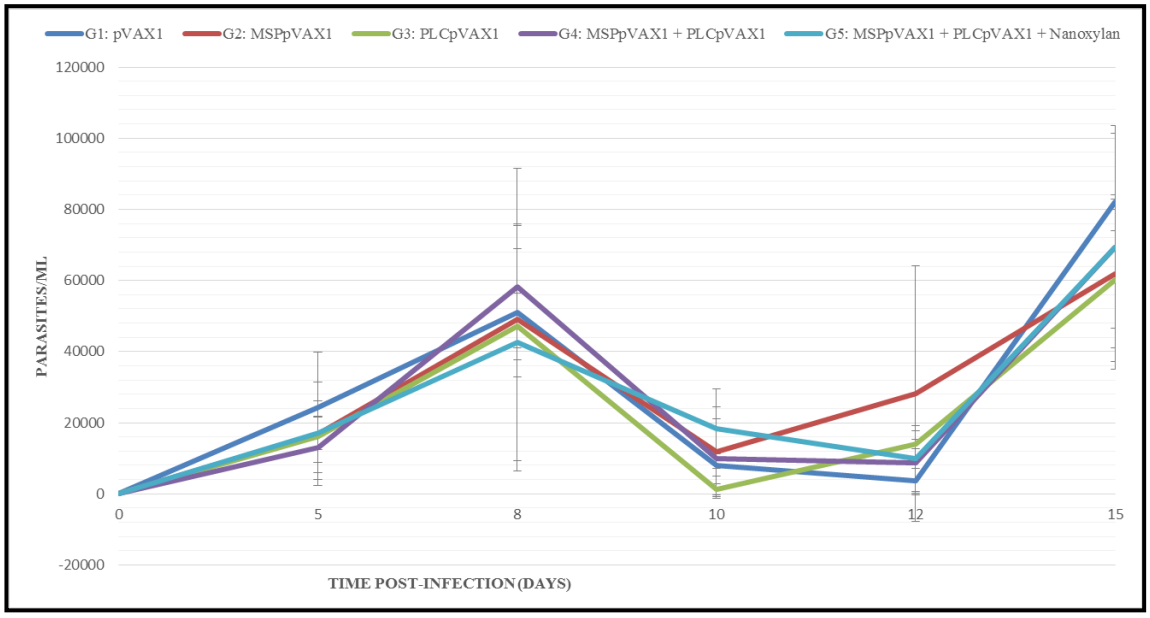

Figure 15: Parasite concentration at days 5, 8, 10, 12 and 15 post-infection with *Trypanosoma brucei brucei*.

In Figure 16, it can be observed that, all animals, except one from the control group, survived until, at least, day 23, and all groups present deaths after 25 DPI. The control group presents 100% of mortality after 27 DPI.

At 27 DPI, the groups presented different mortality rates: G2 and G4 had a mortality of 67%; G3 presented 100% of mortality; and the mortality of G5, was 33%.

The groups G4 and G5 present the highest survival rates, although with slight differences: they reached 67% of mortality at 26 and 28 DPI, respectively. However one of the mice from G4 survived longer (until day 32).

Nevertheless, the groups immunized with the prototype vaccines did not show strong resistance to the infection, when compared with the control group and with some other works in this field, but they survived longer than in a normal infection (without vaccine) (Silva *et al.* 2009; Lança *et al.* 2011). This can be explained with the fact that the pDNA was not strong enough to cause a protective effect on immunized mice or because the challenge assay was done too far apart from the immunization.

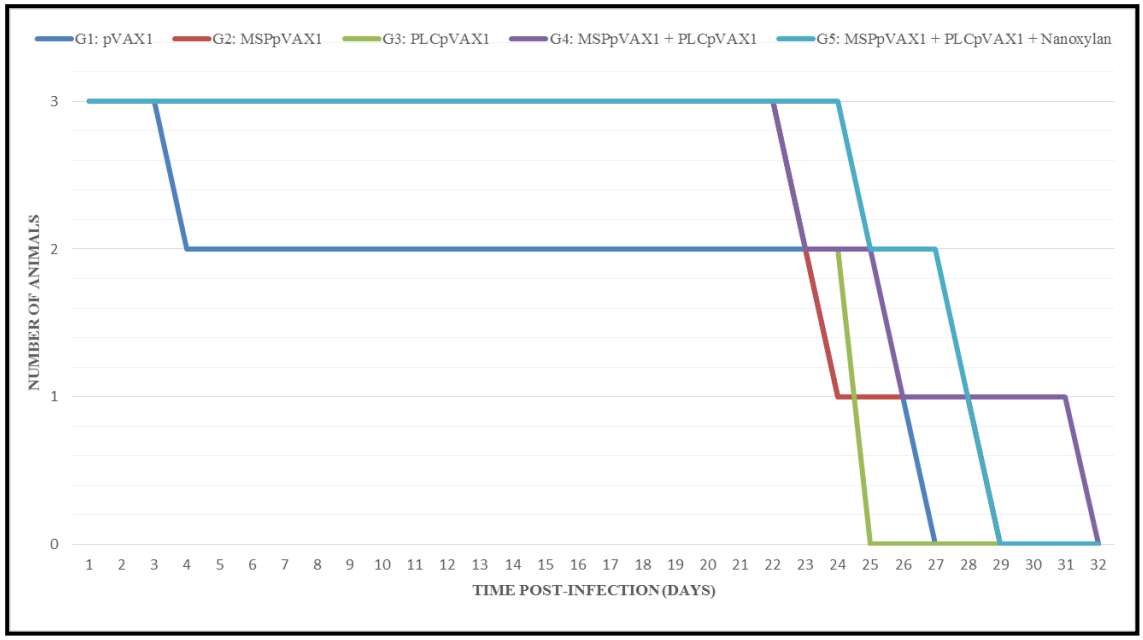

Figure 16: Survival of immunized mice after challenge with *Trypanosoma brucei brucei*.

4.7 Analysis *in silico* of MSP and PLC genes

The resultant sequences from the sequencing with both primers were put together with the help of the software Geneious® (version 8.1.2) (Kearse *et al.* 2012). Then a BLAST was made, in order to determine the sequence most similar to the ones in study and to determine the protein sequence. These results, then allowed the search for additional information about the proteins used, such as molecular weight, theoretical pI, "position" in the cell, the GPI-modification site (Table 6). The zinc motif domain of the MSP was also identified (in Attachment E).

Table 6: Results of an analysis *in silico* of MSP and PLC.

	MSP	**PLC**
Molecular weight (Da)	65689.1	40662.7
Theoretical pI	9.12	8.08
Best-scored position to GPI-modification site	Residue 580 (T)	Residue 335 (K)
Position in the cell	Extracellular	Extracellular

4.8 Design of the antigenic candidates to the VLPs

As mentioned in section 1.5, in this work was used a virus-like particle based on TrV, which is made of 60 copies of a protomer. This protomer is composed by three proteins: VP1, VP2 and VP3 (Figure 17).

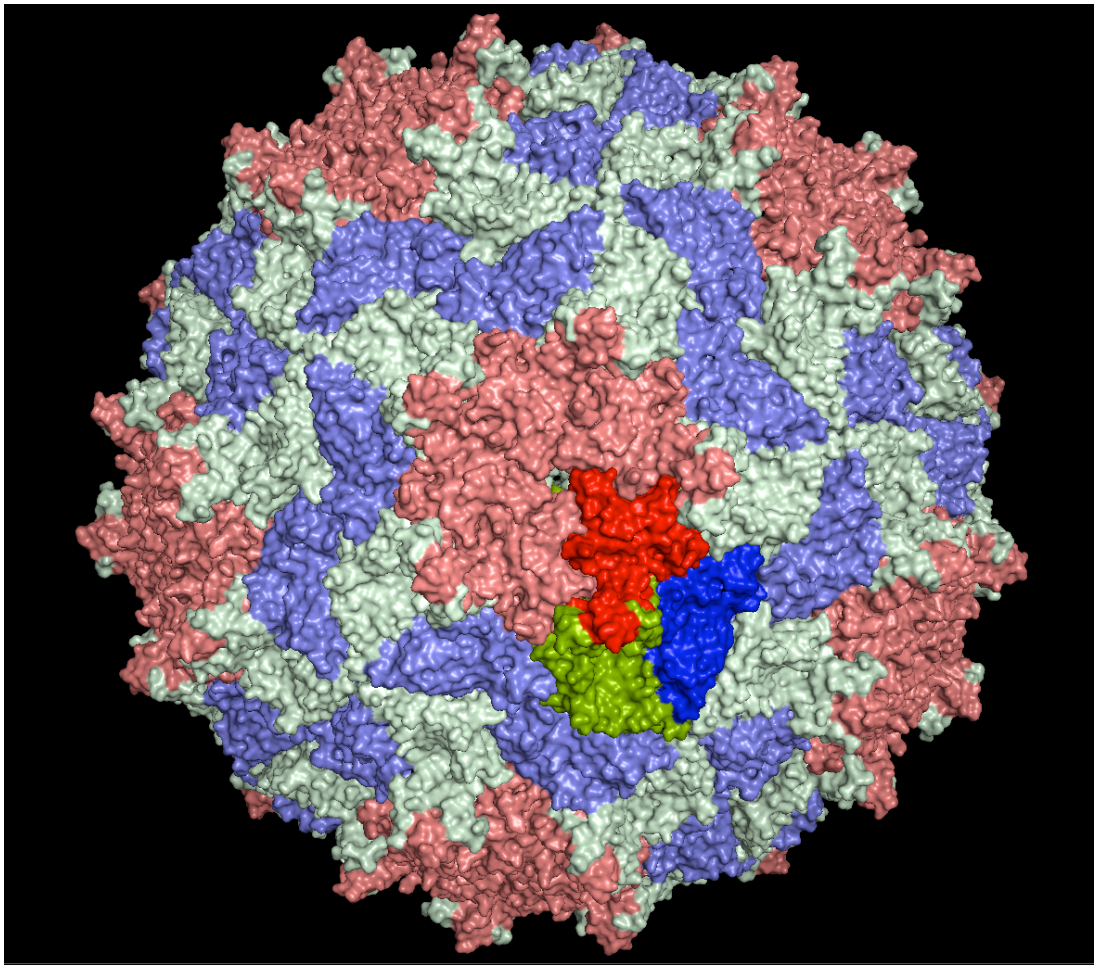

Figure 17: Representation of the capsid of TrV, with the capsid proteins of one protomer highlighted: VP1 – **Red**; VP2 – **Blue**; VP3 – **Green**. Images obtained using PyMOL (Schrodinger LLC 2010).

In order to start the design of the VLP, the first step, as was said before in section 3.16, was the search for possible existence of a tertiary structure of both proteins, MSP and PLC. However, this was not found, because none of the proteins had the tertiary structure resolved. Therefore, was searched for the same proteins but in other organisms, and nothing was found. The alternative was to search for a homolog protein in other organism. So, for the MSP, a homolog protein was found, the GP63 of *Leishmania major* (Schlagenhauf *et al.* 1998). This protein has an identity of 32% with the sequence of the MSP used in this work and both proteins are zinc metalloproteases from organisms from Trypanosomatidae family and the Kinetoplastida order. Besides, GP63 already has antigenic epitopes described, and, as said in the section 1.2.4, the GP63 share positional conservation of 20 cysteines and 10 prolines, with *Trypanosoma brucei* MSP, which suggests common three-dimensional features (LaCount *et al.* 2003). Nevertheless, there was nothing found for the PLC, so this protein could not be used.

After selecting the protein, a bibliographic search for epitopes was done. Among all the epitopes found, only three epitopes were chosen, based on their identity with the MSP used in this work. In Figure 18 is shown the tertiary structure of GP63 and the position of the three chosen epitopes.

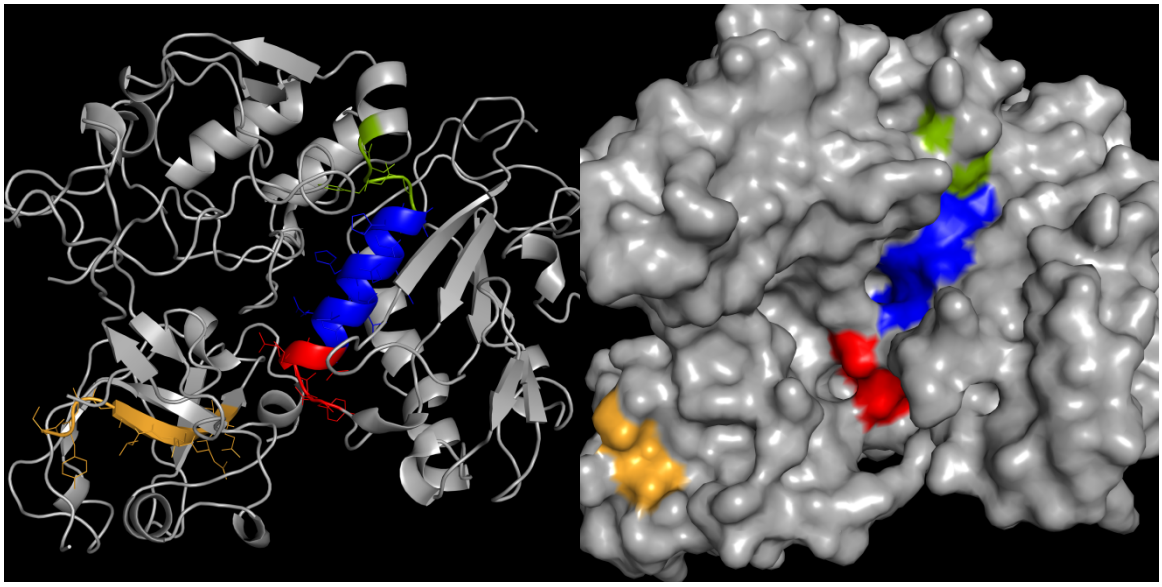

Figure 18: Representation of the epitopes on the tertiary structure of the GP63, ribbon model (left) and surface model (right). The figure with surface shows how the epitopes are exposed. Epi-1 is represented in **red**; Epi-2 is represented in **green**; Epi-3 is represented in **orange**; **blue** represents the common region between Epi_1 and Epi-2. Images obtained using PyMOL (Schrodinger LLC 2010).

The three epitopes chosen were: Epi-1 (Figure 19), Epi-2 (Figure 20) (Russo *et al.* 1993) and Epi-3 (Figure 21) (Elfaki *et al.* 2012). Table 7 shows the relevant information about the three epitopes.

Table 7: Relevant information about the three chosen epitopes from GP63.

Epitope	Region on the sequence	Sequence	Identity with MSP (%)	Tertiary structure
Epi-1	254-269	YDQLVTRVVTHEMAHA	60	α-helix and loop
Epi-2	259-274	TRVVTHEMAHALGFSVG	69	α-helix and loop
Epi-3	503-514	IIKSYAGLCANVQ	83	β-strand and loop

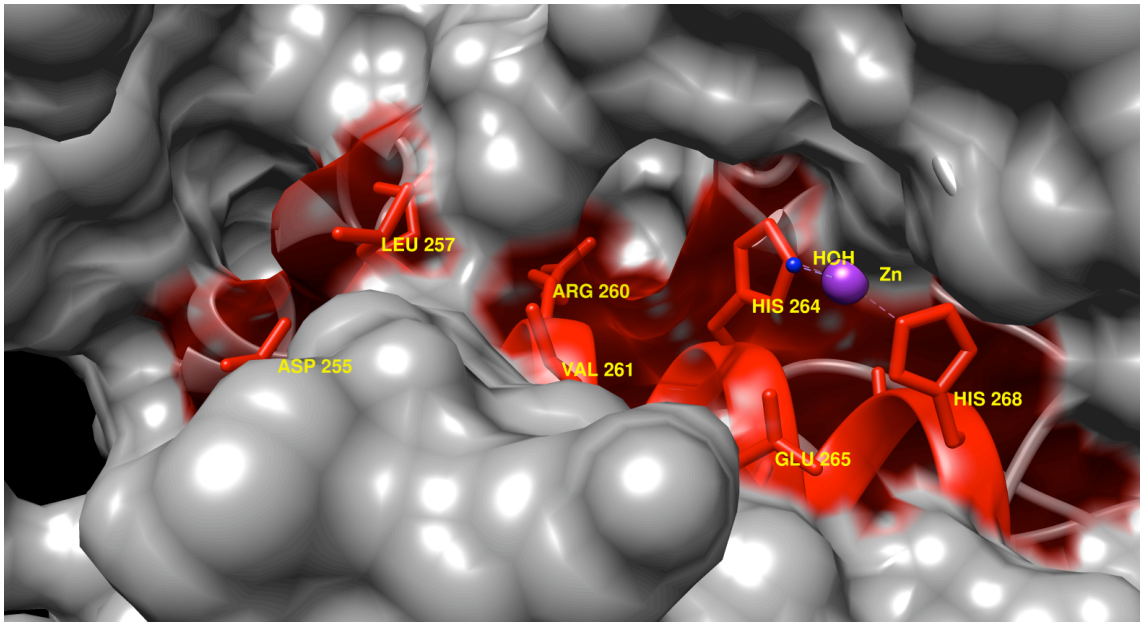

Figure 19: Detailed view of the most exposed amino acids of Epi-1 in the GP63. Images obtained using UCSF Chimera (Pettersen *et al.* 2004).

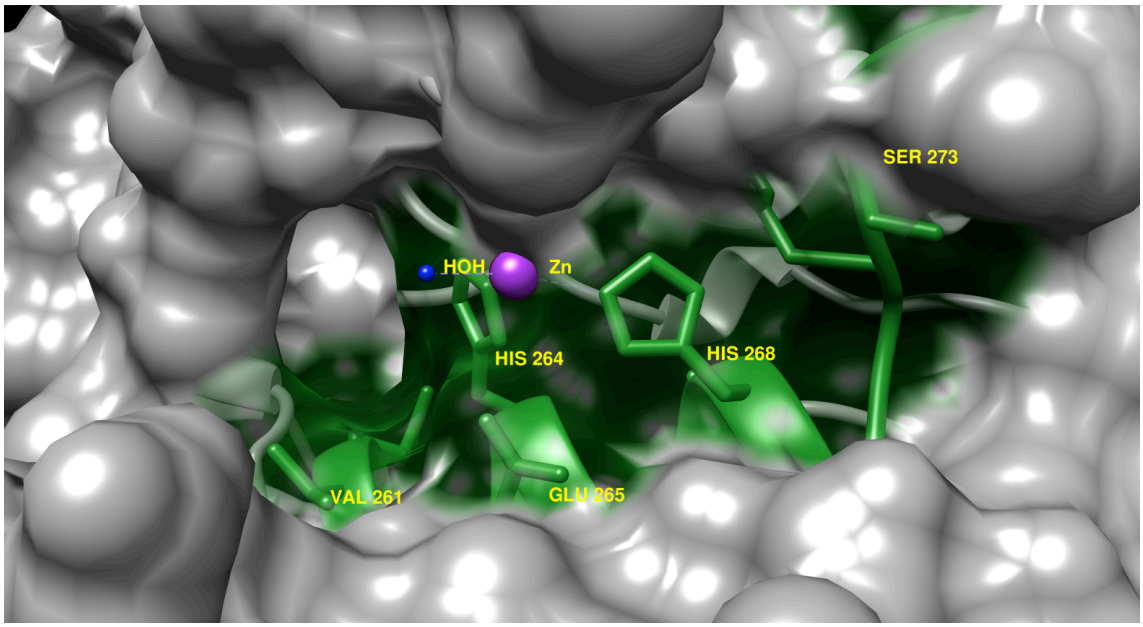

Figure 20: Detailed view of the most exposed amino acids of Epi-2 in the GP63. Images obtained using UCSF Chimera (Pettersen *et al.* 2004).

The tertiary structure of Epi-3 in the GP63 has missing residues due to an uninterpretable weak electron density (Schlagenhauf *et al.* 1998). However, these residues were considered as highly exposed to the solvent (in Attachments F-H) because their supposed position and due to the weak electron density frequently being related to high fluctuation.

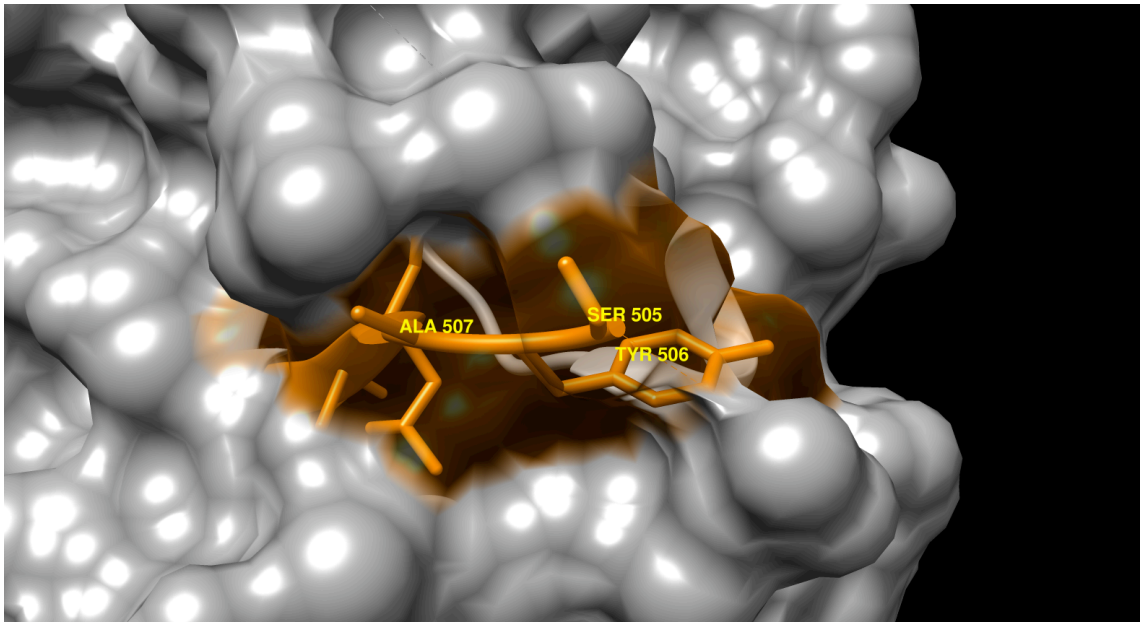

Figure 21: Detailed view of the most exposed amino acids of Epi-3 in the GP63. Images obtained using UCSF Chimera (Pettersen *et al.* 2004).

When all the information about the epitopes had been collected, the TrV structure was analysed and the best mutation sites were selected (Table 8 and Figure 22). Then the replacement of the amino acids from the tertiary structure of the TrV by the amino acids of the epitopes was made, based on the solvent exposure values (in Attachments F-H) and trying to mimic their native form. Both these parameters were the base of the design process: the regions selected in TrV had similar values to the original regions on GP63.

Table 8: Regions of mutation on TrV by epitope.

Epitope	Region of mutation on TrV	Images
Epi-1	94-109 of VP1	23
Epi-2	159-174 of VP2	24
Epi-3	237-249 of VP3	25

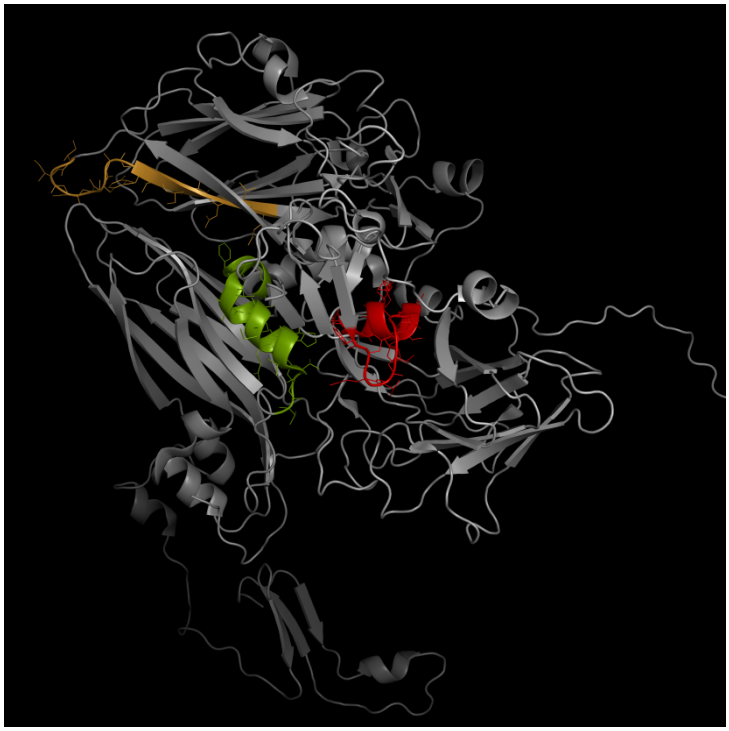

Figure 22: Representation of the TrV protomer with the mutation regions highlighted: **Red** - Epi-1; **Green** - Epi-2; **Orange** - Epi-3. Images obtained using PyMOL (Schrodinger LLC 2010).

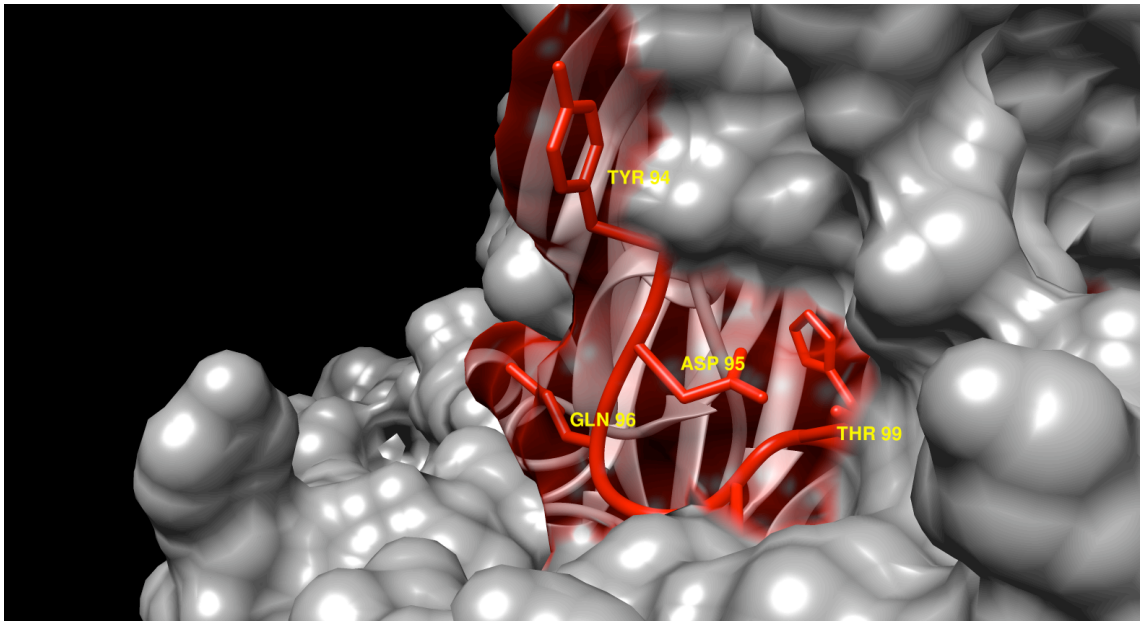

Figure 23: Detailed view of the most exposed amino acids of Epi-1 inserted in TrV. Images obtained using UCSF Chimera (Pettersen *et al.* 2004).

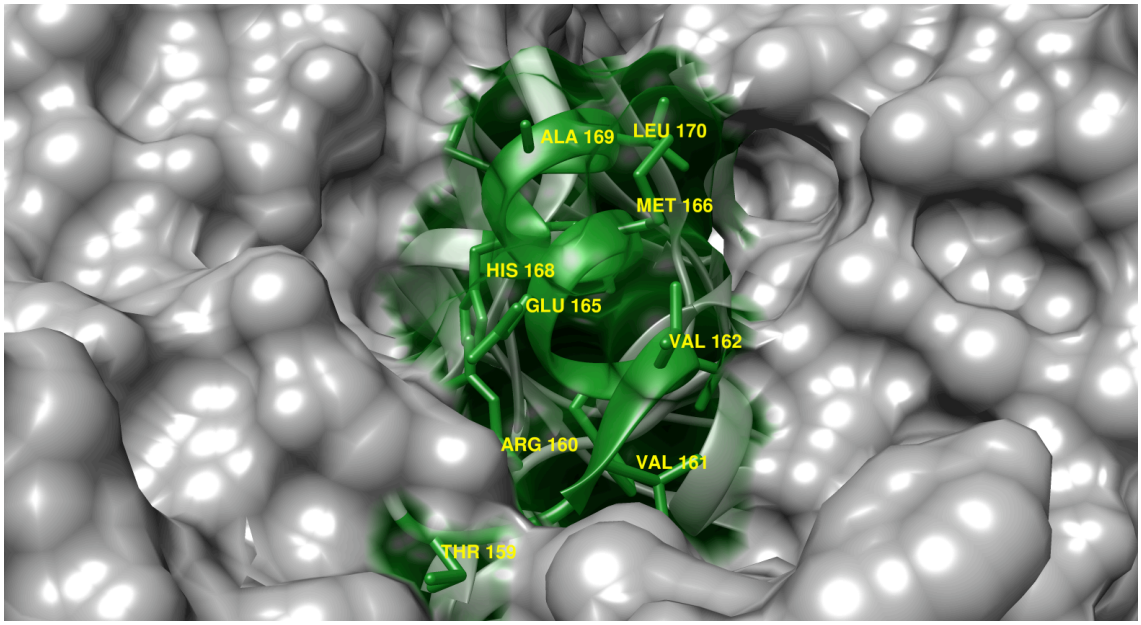

Figure 24: Detailed view of the most exposed amino acids of Epi-2 inserted in TrV. Images obtained using UCSF Chimera (Pettersen *et al.* 2004).

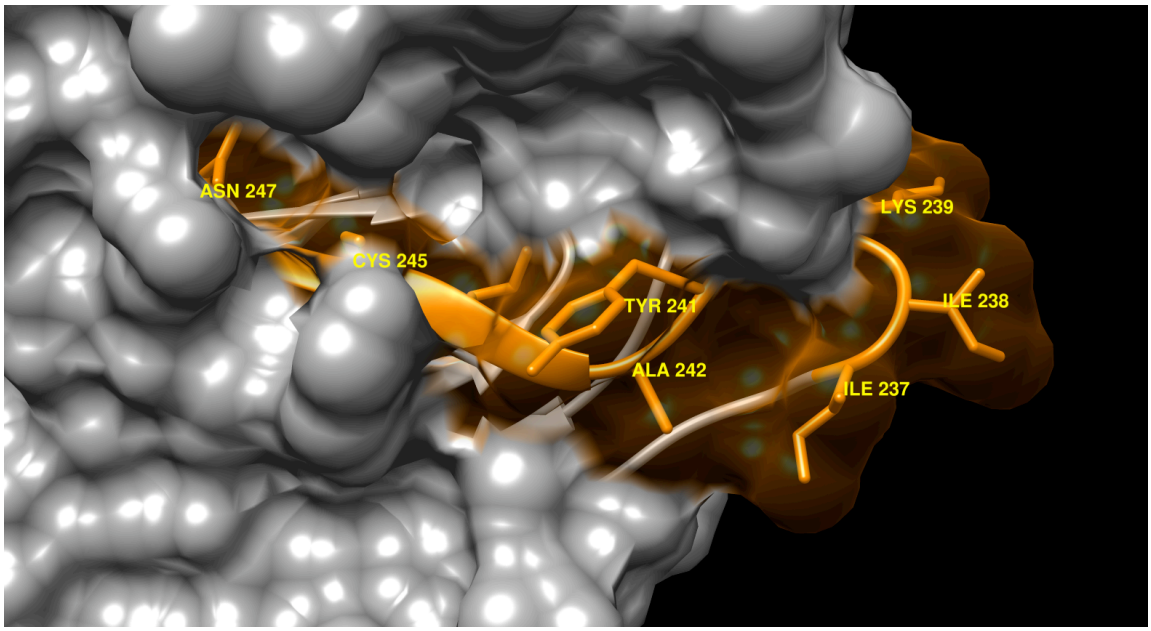

Figure 25: Detailed view of the most exposed amino acids of Epi-3 inserted in TrV. Images obtained using UCSF Chimera (Pettersen *et al.* 2004).

A point to mention about the insertion point for Epi-3, is that when the protomer assembled to form the capsid, the Epi-3s of three protomers were close to each other, as shown in Figure 26 and Figure 28.

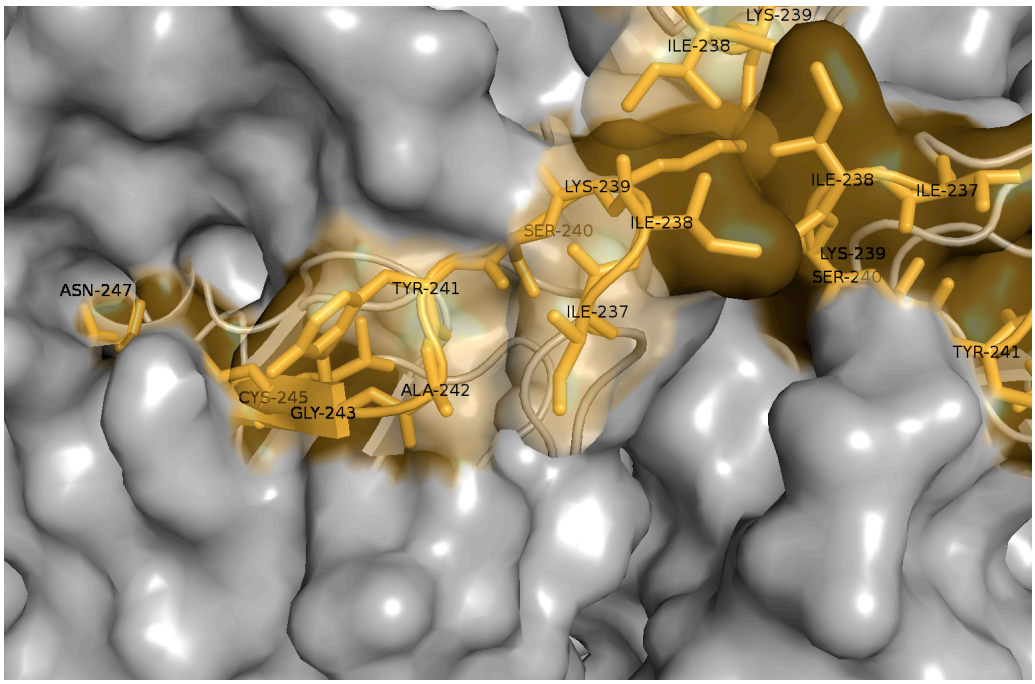

Figure 26: Detailed view of the Epi-3 of three distinct protomers. Images obtained using PyMOL (Schrodinger LLC 2010).

Following the mutation, done in Coot, some of the residues had to be rotated so they would not form undesirable contacts with neighbouring residues. Afterwards the exposition, disulfide bonds and saline bridges were analysed, to see the mutation degree. The exposition was analysed by ASA-View, the disulfide bonds by a script found in pymolwiki and the saline bridge by VMD.

In Epi-1, a saline bridge was found, between residues of the epitope ARG 100 – GLU 105, that was not previously present, *i.e.* in the native structures of GP63 or TrV, however the consequence of this can not be speculated without further molecular dynamics studies.

Another difference found after mutation was on the Epi-3. As said before, GP63 has cysteines, in contrast with TrV, and the Epi-3 had a disulfide bond (Figure 27) that when inserted in TrV was lost. Two possible solutions were conjectured: or add a cystein to the TrV in order to favour the formation of a disulfide bond, or to eliminate the cystein from Epi-3. Nonetheless, because this issue requires more studies, was decided to maintain the cystein in the Epi-3 without doing any additional change into TrV structure.

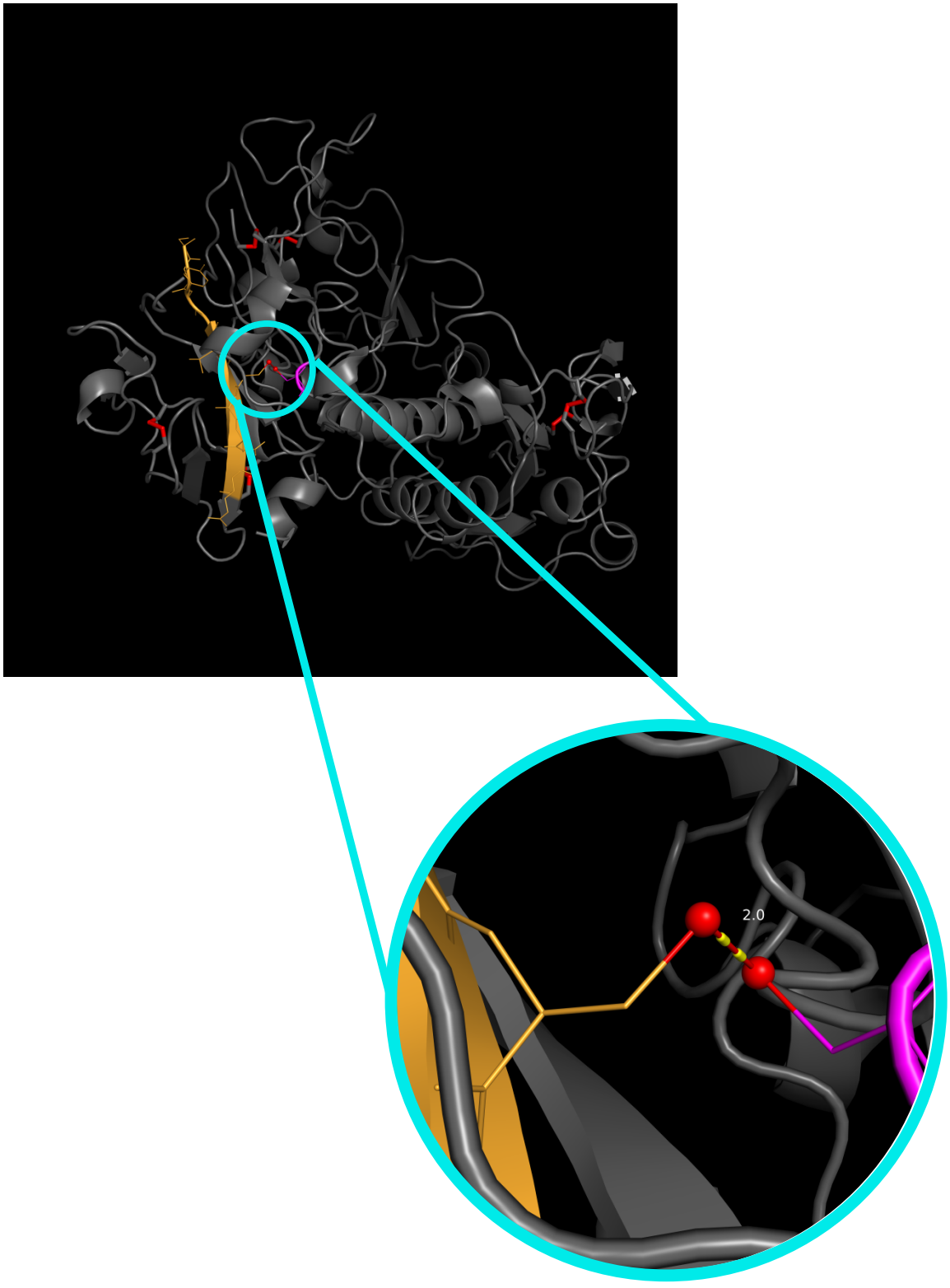

Figure 27: Detailed view of the disulfide bond in GP63, before mutation. Images obtained using PyMOL (Schrodinger LLC 2010).

Finally, when all necessary changes were done the result was a VLP based on TrV with 3 epitopes from GP63 of *Leishmania major*. In Figure 28, the final result of the design is shown. It is distinguishable the pentamere (light grey) and all the epitopes: Epi-1 in red, Epi-2 in green and Epi-3 in orange. Besides, it is possible to have a general view of the overlap between the Epi-3 of different protomers.

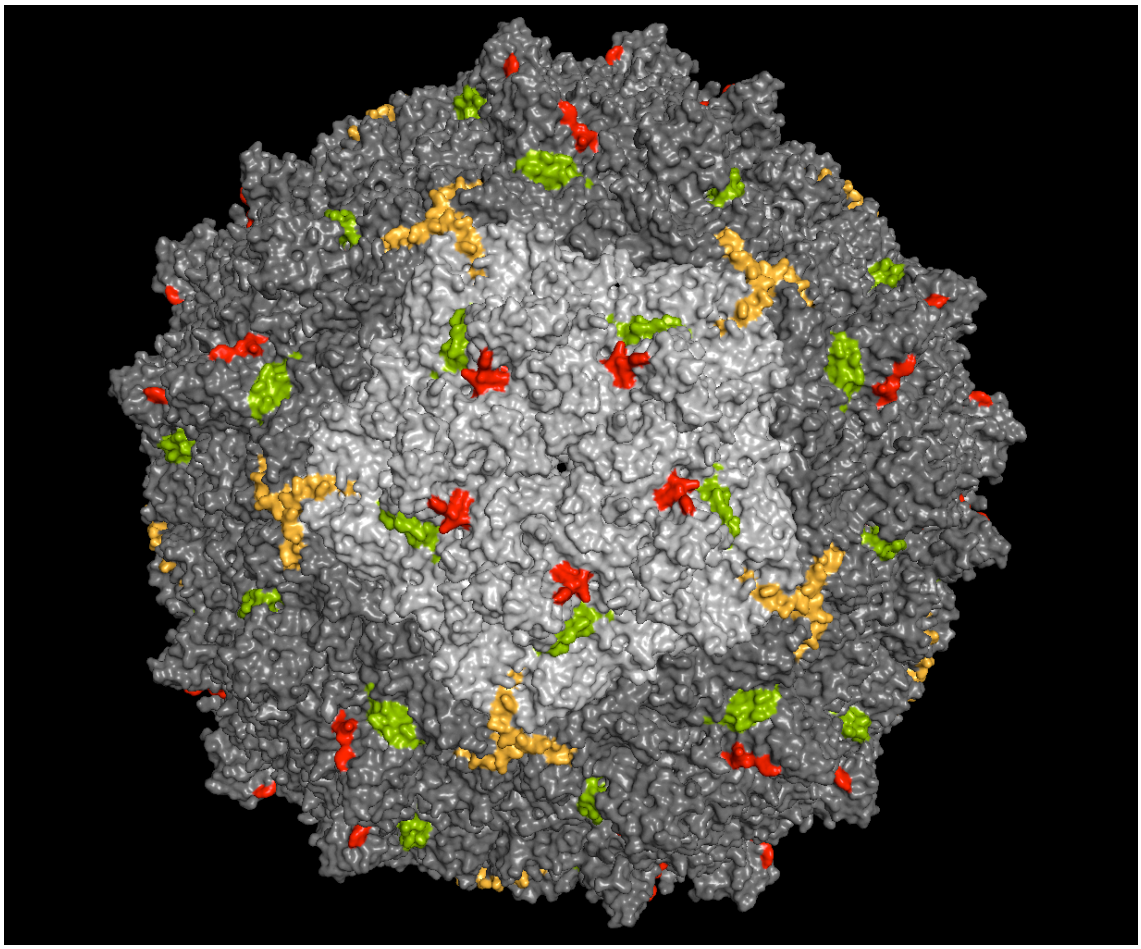

Figure 28: Final result: Capsid of TrV with the three epitopes inserted: Epi-1 - **Red**; Epi-2 - **Green**; Epi-3 - **Orange**. Images obtained using PyMOL (Schrodinger LLC 2010).

5. Conclusions and Future Perspectives

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The work developed had the main objective of elaborating different models of DNA immunization as a strategy for vaccination against African Trypanosomiasis. The results obtained were analyzed and discussed taking into account previous works in this field, even though, the DNA vaccination against this disease has been poorly studied.

1- Cloning the genes MSP and PLC of the bloodstream forms of *Trypanosoma brucei brucei* in pVAX1 and pET28a: All of the plasmids were successfully cloned and characterized by restriction analysis, however, when it comes to automated sequencing, the PLCpET28a was the only one that was impossible to obtain a sequence.

2- Use the plasmids MSPpET28a and PLCpET28a to express the recombinant protein of MSP and PLC in *Escherichia coli*: Although several conditions were used to achieve this goal, none of the recombinant proteins were expressed, possibly due to the fact that the proteins in study are membrane proteins with partially hydrophobic surfaces, flexibility and lack of stability. In order to overcome this issue and to accomplish the expression of the recombinant proteins, it would be crucial to amplify the extracellular portion of the genes.

3- *In silico* analysis and design the epitopes as candidates to the VLPs: With the analysis *in silico*, it was possible to collect some additional information about the proteins being used, like, for example, both proteins are extracellular and the zinc motif domain of the MSP was identified. Additionally, the analysis facilitated the search for a homologous of the MSP, in order to make the design of the VLPs, since it was not possible to use the original proteins, once that they have no tertiary structure or antigenic epitopes described. Regarding this model of immunization, the next step would be a biological evaluation of the VLP designed.

4- Use the plasmids MSPpVAX1 and PLCpVAX1 as model to the development of DNA vaccines against *Trypanosoma brucei*, determine anti-*Trypanosoma brucei* antibodies and identify native proteins with serum from immunized mice by DNA vaccine, and challenge the mice with a lethal dose of *Trypanosoma brucei brucei*: The plasmids MSPpVAX1 and PLCpVAX1 were used as models of DNA vaccines, with 4 different compositions, the immunization protocol was followed, and the antibodies anti-*Trypanosoma brucei brucei* were measured and, although the titres were relatively low, they were more evident in groups immunized with PLCpVAX1, MSPpVAX1+PLCpVAX1 and MSPpVAX1+PLCpVAX1+nanoxylan, the last one presented higher titres, which probably means that the nanoxylan works as an adjuvant. However, the proteins identified did not have the expected molecular weights of MSP and PLC, and the intensity of the bands suggests low levels of protein expression in the mice. Although none of the immunized mice was able to control the infection, they were able to survive longer to it. In the future, some adjustments to the present prototypes are needed, such as: increase the number of animals used, increase the number of plasmids injections, optimize the immunization times, increase the amount of plasmid and nanoxylan injected and try new adjuvants.

6. References

6. References

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7. Attachments

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Solution P1 - Alkaline lysis

-10 mM EDTA

- 50 mM Glucose

- 25 mM Tris-HCl pH 8.0

Solution P2 - Alkaline lysis

- 200 nM NaOH

- 1% SDS

Solution P3 - Alkaline lysis

- 3M Potassium Acetate pH 5.0

Solution 1 - HIC

- 10 mM Tris-HCL pH 8.0

- Water MQ sterile

Solution 2 - HIC

- 1.5 M Ammonium Sulphate; Prepared in Solution 1

TBS 10X (Triphosphate Buffer Saline)

- 50 mM Tris-HCl pH 8.0

- 150 mM NaCl

- Deionized Water *q.s.* 1.0 L; pH 7.5

PBS 10X (Phosphate Buffer Saline)

- 27 mM de KCl

- 1,37 M de NaCl

- 17,6 mM de KH2PO4

- 101,4 mM de Na2HPO4

- Deionized Water *q.s.* 1,0 L; pH 7,4.

Bicarbonate Buffer 0.1 M pH 8.5

- 0,1 M de $NaHCO_3$

Citrate Buffer pH 5.0

- 24,3 mL Citric Acid 0.1 M

- 25,7 mL Sodium Phosphate 0.1 M

- Sterile Water *q.s.* 100 mL

Attachment A- Results of automated sequencing of MSP in pVAX1.

ATGACCCAACTGTTAGGAACCGCTAACTTTTGGTGCATATTTGCCGCTTTC
GTCTCGCACCACTTGCGAGCACACGTGCACGTGGAAGCATCAGCGACACA
TTTGGAAGCACCAGAGGAACAATGGGGAGAGGAAGGCACCGGCGATACG
CCCCGTGGTTGGTGCGGAAGTCATCATTCTGCGATTAATCCAGATGATGTG
CCGATAGTAGGTACTATGCCCCCTGAATCAGAAGCGAAGGGTACCACTGG
CGGTGATTTGATTAGTGCAAGAACTGCATCCGTAGATAAGAAACCTAAAT
ACACCAATAACGTTGATGATTACGGGCAAGGGGAGATTGACAGCAGGTGG
AAACCAATTCGCATCAGAGCTTACACGCAAGACTTGAATGACCCCAGCCG
CTTCTGCACGATGGCAGGTGATGTCCGTAGCATACTCGTCAGTGGGAAGA
CGACTGTTTGTACAGCGGGGGATGTTCTCACAGTTCGCAAGAAGCGCGTC
ATCGTGCAGGTCGCTATTCCAAAGGCAATCAAATTACACACGGATCGCCT
GTTGGTAAGGAGATATCACCGGAGGATTGTTTTACCATCCTCATACGCTGG
ATACTGCAGCTTATTCAAGGTGCCCAAGGGTCACTACACCAATGGATTTGA
AGGTGATGTTAGTATATACGTGGCGGCTCGGCCAACGATAGGGAATATGG
CGTGGGCTTCCGTGTGCGCAATGCTCACTGACGGCCGCCCGGTTTCTGGCG
TTGTCAACATATCCCCCAAGTACGTTGCCGAAACTGACTTCTTTGTGCGAG
TTATCGCACACGAATTAGGACACGCTCTCGGCTTCCAAGCAGATATCCTCA
TAAGAAGGGGAATTATGAAACAGAAAGGAGGCATTCGCGGACTTAGAAC
CTCGTGGTTGGTCGATTCTGAGGTTGCGAAGCGTGTCCCGCGGAAGCACTT
CAATTGCTCGACGGCTCCGGGTATCGAAATGGAGAATGAAGGAGGCCCCG
GCGTTTTCGCAACACACTTGGAGCAACGCAATGCCGTCTAGGACGTGATG
GCCCCTTATGGGAACTTGAATAATTTAACTGTGATGTCGTTAGGGGTGTTC
GCAAGTATGGGACACTACCGCGTCAATTTCAGTCGCGCAGAGAAGACGCG
TTGGGGCCTAAATCGTGGATGTAGTTTTCTACAGGAGAAATGCTTGCAGGA
AGGAAAGTCAAAGCACCCCGACACGTTCTGCGATCATTTATGGAAGAGCC
GTCTGTTCACTTGTACCCATGACCGTCTCGGACTCGGCCAGTGTTCCCTAG
GTACACATCGAACTGAACTTCCCGCCGAGTTCCGCTACTTCAGAAATTCAC
GGGTTGGTGGCAAGTCCCGTTTCATGGACCATTGCCCGATGGTGGTTCAGT
ACAACAGCGGTAACTGCGTCAATGGTCAGTCGAAGTTTTTGCGTGGCAGT
GAGGTTGGAAAGGGCTCGCGATGTGTGAAGGGTGTCAATTTGAAGTTTTC
CAATAAAGATATTGGTGACGTTTGTGTCCGTACGAACTGCACTGGCAAAG
AGCTACAGATTCGCTTTTTGCTAGACCACAGCTGGCAGACGTGTAAACCCG
GTGCTACAGTGCAGCCCCTCGGTCGTCACCTCTGGAAAGGCAGCATCATCT
GCCCCACGCGGGAAGAAGTTAGTTTCGATGACGAGGATTACAAGCTTAGA
TTGACCCCACTTCCGAAACTCCCCACTGACGACAACGCGGCCGTGAACCCT
CGTCAAATGTGA

Attachment B - Results of automated sequencing of MSP in pET28a.

ATGACCCAACTGTCAGGAACCGCCATCTTTTGGTGCATATTTGCTGCTTTCG
TCTCGCACCACTTGCGAGCACACGTGCACGTGGAAGCATCAGCGACACATT
TGGAAGCGCCAGAGGAACAATGGGGAGAGGAAGGCACCGGCGATACGCCC
CGTGGTTGGTGCGGAAGTCATCATTCTGCGATTAATCCAGATGATGTGCCGA
TAGTAGGTACTATGCCCCCTGAATCAGAAGCGAAGGGTACCACTGGCGGTG
ATTTGATTAGTGCAAGAACTGCATCCGTAGATAAGAAACCTAAATACACCA
ATAACGTTGATGATTACGGGCAAGGGGAGATTGACAGCAGGTGGAAACCA
ATTCGCATCAGAGCTTACACGCAAGACTAGAATGACCCCAGCCGCTTCTGC
ACGATGGCAGGTGATGTCCGTAGCATACTCGTCAGTGGGAAGACGACTGTT
TGTACAGCGGGGGATGTTCTCACAGTTCGCAAGAAGCGCGTCATCGTGCAG
GTCGCTATTCCAAAGGCAATCAAATTACACACGGATCGCCTGTTGGTAAGG
AGATATCACCGGAGGATTGCTTTACCATCCTCATACGCTGGATACTGCAGCT
TATTCAAGGTGCCCAAGGGTCACTACACCAATGGTTTTGAAGGTGATGTTAG
TATATACGTGGCGGCTCGGCCAACGATAGGGAATATGGCGTGGGCTTCCGT
GTGCGCAATGCTCACTGACGGCCGCCCGGTTTCTGGCGTTGTCAACATATCC
CCCAAGTACGTTGCCGAAACTGACTTCTTTGTGCGAGTTATCGCACACGAAT
TAAGGACACGCTCTCGGCTTCCAAGCAGATATCCTCATAAGAAGGGGATTT
ATGAAACAGAAAGGAGGCATTCCCGGACTTAAAACCTCGTGGGTGGGCGAT
TCTGAGGTTGCGAAGCGTGTCGCGCGGAAGCACTTCAATTGCTCGACGGCT
CCGGGTATCGAAATGGAGAGTGAAGGAGGCCCCGGCGTTTTCGCAACACAC
TTGGAGCAACGCAATGCCGTCGAGGACGTGATGGCCCCTTATGGGAACTTG
AATTATTTTAACTGTGATGTCGTTAGGGGTGTTCGCAAGTATGGGACACTAC
CGCGTCAATTTCAGTCGCGCAGAGAAGACGCGTTGGGGCCTGAATCGTGGA
TGTAGTTTTCTACAGGAGAAATGCTTGCAGGAAGGAAAGACAAAGCACCCC
GACACGTTCTGCGATCATTTATGGAAGAGCCGTCTGTTCACTTGTACCCATG
ACCGTCTCGGACTCGGCCAGTGTTCCCTAGGTACACATCGAACTGAACTTCC
CGCCGAGTTCCGCTACTTCAGAAATTCACGGGTTGGTGGCAAGTCCCGTTTC
ATGGGCCATTGCCCGATGGTGGTTCAGTACAACAGCGGTAACTGCGTCAAT
GGTCAGTCGAAGTTTTTGCGTGGCAGTGAGGTTGGAAAGGGCTCGCGATGT
GTGAAGGGTGTCAATTTGAAGTTTTCCAATAAAGATAATGGTGACGTTTGTG
TCCGTACGAACTGCACTGGCAAAGAGCTACAGATTCGCTTTTTGCTAGACCA
CAGCTGGCAGACGTGTAAACCCGGTGCTACAGTGCAGCCCCTCGGTCGTCA
CCTCTGGAAAGGCAGCATCATCTGCCCCACGCGGGAAGAAGTTTGTTTCGA
TGACGAGGATTACAAGCTTAGATTGACCCCACTTCCGAAACTCCCCACTGAC
GACAACGCGGCCGTGAACCCTCGTCAAATGTGA

Attachment C - Results of automated sequencing of PLC in pVAX1.

ATGTTTGGTGGTGTAAAGTGGTCACCGCAGTCATGGATGAGTGACACGCGG
TCTTCCATTGAGAAGAAATGTATTGGTCAAGTATATATGGTAGGAGCACAT
AATGCAGGAACACACGGCATACAAATGTTTTCCCCGTTTGGATTAGATGCCC
CTGAAAAGTTACGGAGCCTCCCTCCATATGTGACCTTTCTTTTAAGATTTCTT
ACTGTTGGTGTGAGCAGCAGATGGGGACGTTGTCAAAATCTTTCTATTCGAC
AGCTTTTGGATCATGGGGTGCGTTATCTCGACTTACGCATGAACATAAGTCC
GGATCAGGAAAATAAAATTTACACAACGCATTTCCATATTTCTGTTCCACTA
CAAGAGGTTCTGAAGGATGTCAAGGATTTCTTGACCACACCTGCAAGCGCC
AACGAATTTGTCATCCTCGATTTCTTGCATTTCTACGGATTTAATGAGAGCC
ATACGATGAAGCGCTTTGTTGAGGAGCTGCAAGCACTTGAGGAGTTTTACA
TTCCCACAACCGTCTCTTTAACCACACCACTTTGTAACCTCTGGCAGTCAAC
CAGACGTATTTTTCTTGTTGTGAGACCTTATGTAGAATACCCTTATGCACGA
CTCCGCAGTGTTGCGCTTAAATCCATTTGGGTTAATCAAATGGAGTTGAATG
ATCTTCTCGACCGGTTGGAGGAACTCATGACGCGTGATTTGGAAGATGTCA
GTATTGGCGGGGTTCCATCTAAAATGTACGTCACGCAAGCTATCGGTACGCC
GCGAAATAACGACTTTGCGGTGGCAGCGTGTTGTGGCGCGTGTCCCGGTTC
ACATCCCGATTTGTATTCCGCTGCAAAGCATAAAAATCCACATCTTTTGCAG
TGGTTTTATGATTTAAATGTTAATGGTGTTATGCGAGGGGAGCGTGTGACTA
TAAGACGGGGAAACAATACACATGGAAATATACTTTTGCTTGATTTCGTGC
AAGAAGGCACTTGTACTGTTAAGGGAGTTGACAAACCGATGAATGCCGTTG
CATTATGCGTTCATTTAAACACCAACCAAACCGCAAGGTCATAAGGATCCA
CTAGTCCAGGGGGGTGGAATTCTGCAAATATCCGCACAGTGGCGGCCGCTC
CAATTTAAAGGGCCCGTTAAACCCGTTGATCAGCCT

Attachment D - Protein sequence of PLC.

MFGGVKWSPQSWMSDTRSSIEKKCIGQVYMVGAHNAGTHGIQMFSPFGLDAP
EKLRSLPPYVTFLLRFLTVGVSSRWGRCQNLSIRQLLDHGVRYLDLRMNISPDQ
ENKIYTTHFHISVPLQEVLKDVKDFLTTPASANEFVILDFLHFYGFNESHTMKRF
VEELQALEEFYIPTTVSLTTPLCNLWQSTRRIFLVVRPYVEYPYARLRSVALKSI
WVNQMELNDLLDRLEELMTRDLEDVSIGGVPSKMYVTQAIGTPRNNDFAVAA
CCGACPGSHPDLYSAAKHKNPHLLQWFYDLNVNGVMRGERVTIRRGNNTHGN
ILLLDFVQEGTCTVKGVDKPMNAVALCVHLNTNQTARS

Attachment E - Protein sequence of MSP with the zinc motif highlighted.

MTQLLGTAIFWCIFAAFVSHHLRAHVHVEASATHLEAPEEQWGEEGTGDTPRG
WCGSHHSAINPDDVPIVGTMPPESEAKGTTGGDLISARTASVDKKPKYTNNVD
DYGQGEIDSRWKPIRIRAYTQDLNDPSRFCTMAGDVRSILVSGKTTVCTAGDVL
TVRKKRVIVQVAIPKAIKLHTDRLLVRRYHRRIVLPSSYAGYCSLFKVPKGHYT
NGFEGDVSIYVAARPTIGNMAWASVCAMLTDGRPVSGVVNISPKYVAETDFFV
RVIA**HELGHALGF**QADILIRRGIMKQKGGIRGLKTSWLVDSEVAKRVARKHFN
CSTAPGIEMENEGGPGVFATHLEQRNAVEDVMAPYGNLNYLTVMSLGVFASM
GHYRVNFSRAEKTRWGLNRGCSFLQEKCLQEGKSKHPDTFCDHLWKSRLFTCT
HDRLGLGQCSLGTHRTELPAEFRYFRNSRVGGKSRFMDHCPMVVQYNSGNCV
NGQSKFLRGSEVGKGSRCVKGVNLKFSNKDIGDVCVRTNCTGKELQIRFLLDH
SWQTCKPGATVQPLGRHLWKGSIICPTREEVCFDDEDYKLRLTPLPKLPTDDNA
AVNPRQM

Attachment F - Exposition values of Epi-1.

Residue	Exposition on GP63	Exposition on mutated TrV
Y	0.140370	0.608270
D	0.200825	0.422425
Q	0.022396	0.778261
L	0.311277	0.032766
V	0.123614	0.006506
T	0.043254	0.086508
R	0.061124	0.117882
V	0.130120	0.013012
V	0.000000	0.104096
T	0.000000	0.064881
H	0.098946	0.005497
E	0.183168	0.017172
M	0.004997	0.024985
A	0.000000	0.000000
H	0.115437	0.032982
A	0.000000	0.063518

Attachment G - Exposition values of Epi-2.

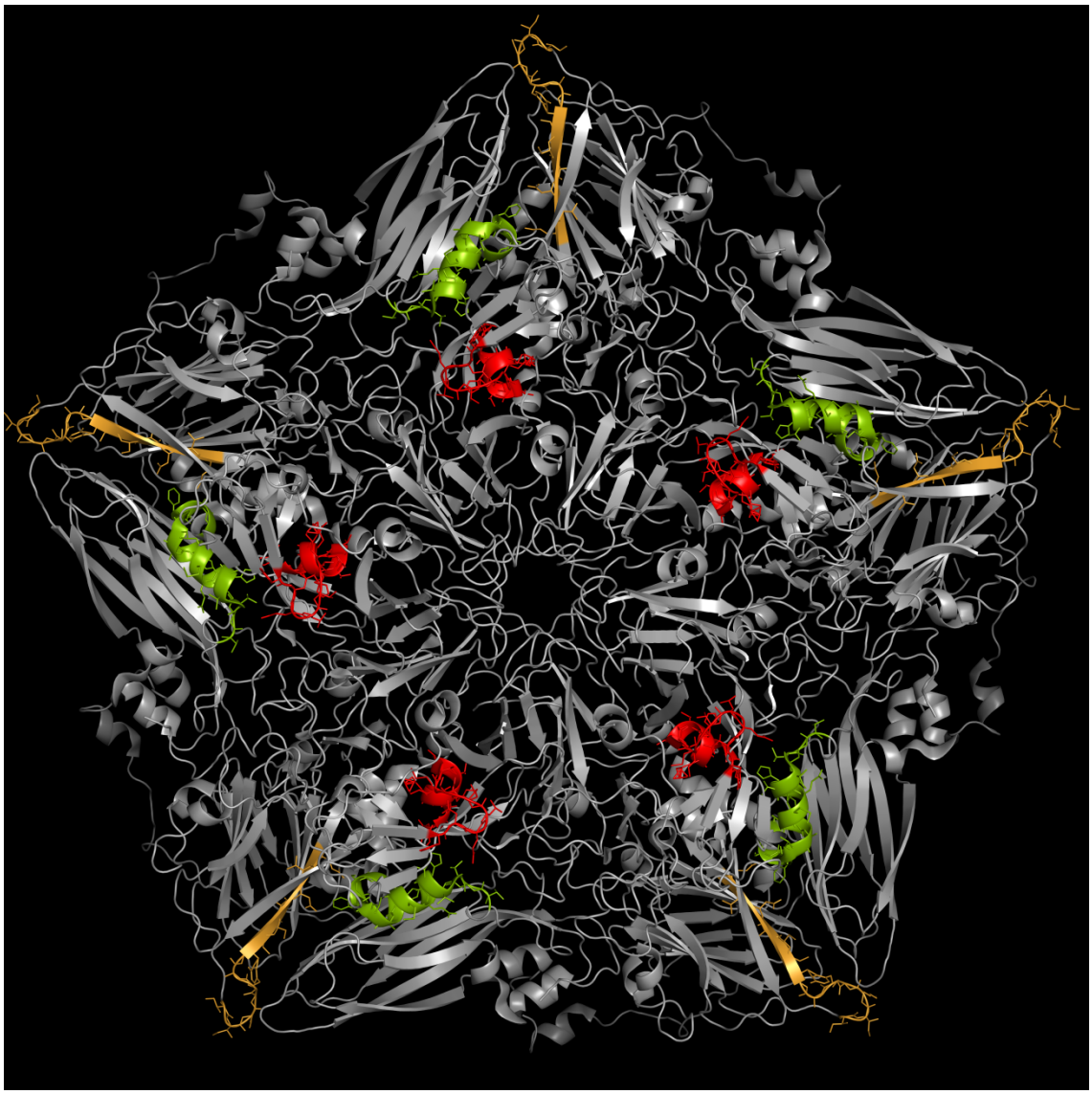
Residue	Exposition on GP63	Exposition on mutated TrV
T	0.043254	0.036045
R	0.061124	0.113516
V	0.130120	0.097590
V	0.000000	0.611564
T	0.000000	0.014418
H	0.098946	0.071461
E	0.183168	0.400680
M	0.004997	0.434739
A	0.000000	0.226850
H	0.115437	0.082455
A	0.000000	0.617032
L	0.005461	0.305816
G	0.000000	0.216002
F	0.004982	0.000000
S	0.162108	0.179172
G	0.114354	0.216002

Attachment H - Exposition values of Epi-3.

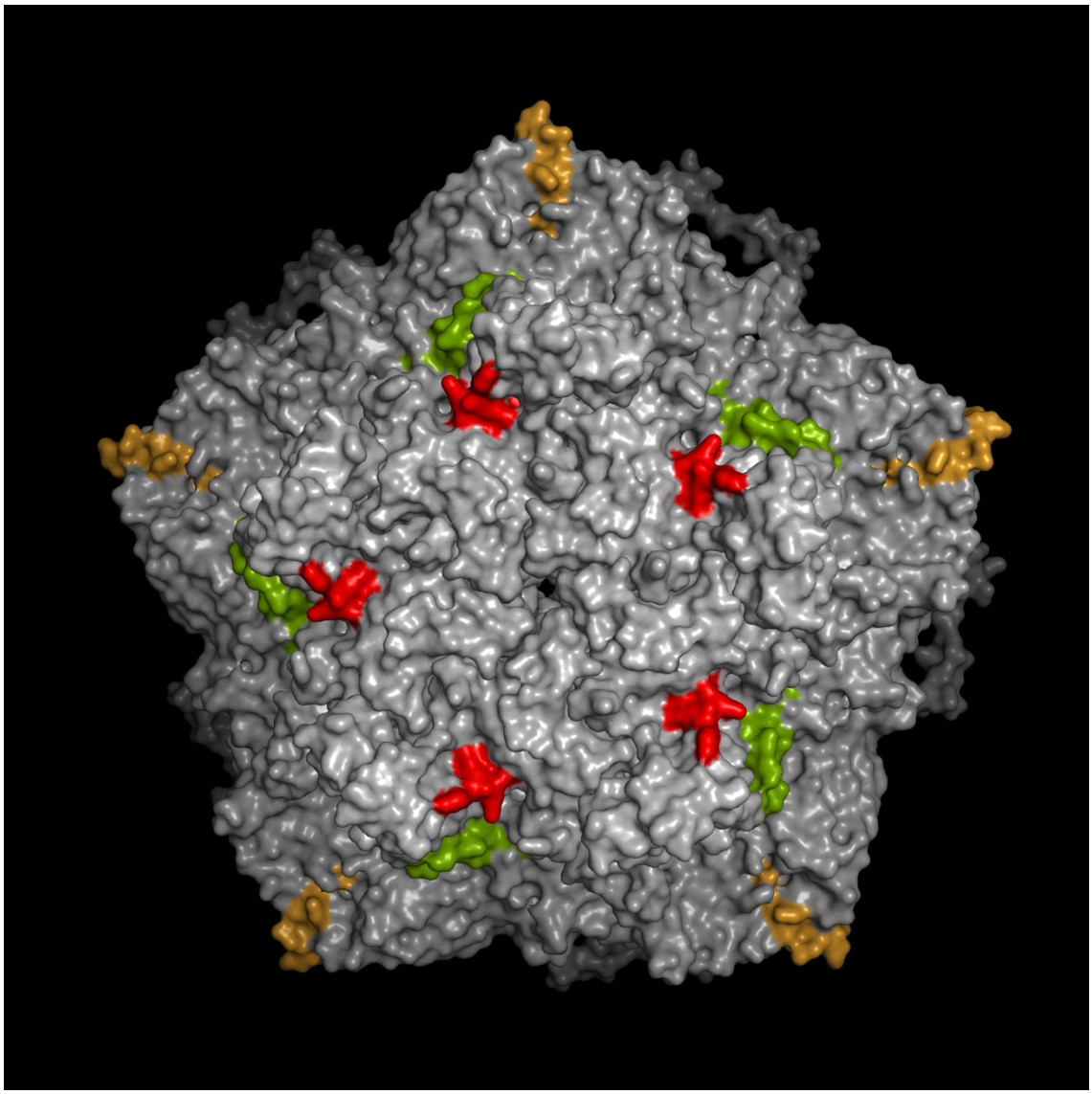
Residue	Exposition on GP63	Exposition on mutated TrV
I	*	0.691840
I	*	0.810750
K	*	0.549293
S	0.767880	0.017064
Y	0.262024	0.463221
A	0.117962	0.217776
G	0.000000	0.025412
L	0.000000	0.043688
C	*	0.104085
A	0.000000	0.009074
N	0.116110	0.006830
V	0.006506	0.000000
Q	0.279950	0.027995

* These residues are not described in the tertiary structure.

Attachment I – Ribbon model of the TrV pentamer with the mutation regions highlighted: **Red** – Epi-1; **Green** – Epi-2; **Orange** – Epi-3. Images obtained using PyMOL (Schrodinger LLC 2010).



Attachment J - Surface model of the TrV pentamer with the mutation regions highlighted: **Red** – Epi-1; **Green** – Epi-2; **Orange** – Epi-3. Images obtained using PyMOL (Schrodinger LLC 2010).



Attachment K – Ribbon model of the capsid of TrV with the three epitopes inserted: **Red** – Epi-1; **Green** – Epi-2; **Orange** – Epi-3. Images obtained using PyMOL (Schrodinger LLC 2010).